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Altered T Cell-Mediated Immunity and Infectious Factors in Autism

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ALTERED T CELL-MEDIATED IMMUNITY AND INFECTIOUS FACTORS IN AUTISM

by

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ABSTRACT

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by

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Three major questions were addressed in this dissertation: 1) Do immune abnormalities associated with autism primarily alter CD4+ T cell-mediated or humoral immune responses? 2) Are specific T cell clones expanded in autism? 3) Which, if any, infectious agents play a role in autism?

CD4+ T cell-mediated (Th1) or humoral (Th2) immune responses can be distinguished on the basis of the cytokines expressed. CD4+ T-cells secrete interleukin type 2 (IL-2) and interferon-γ, whereas a Th2 response is associated with secretion of interleukin type 4 (IL-4). mRNA extracted from peripheral blood mononuclear nuclear cells (PBMC) showed significantly increased levels of IL-2 and interferon-γ expression in 24 autistic subjects relative to 19 normal controls. IL-4 mRNA was undetectable in the same group of autistic subjects. These results indicate that a CD4+ T cell-mediated immune response is associated with autism.

The expression of V-β chain mRNA was used as a marker of particular T cell clone expression. The expression of V-β 13 was significantly elevated in the study group

of 11 autistic subjects, but not in 9 normal subjects. This suggests that T cell-mediated autoimmunity is a factor in the disease. Two types of human leukocyte antigens (HLA) alleles, DR4 and DR1, are associated with autism. The association between V-β 13 expressing T cell clones and autism was shown even more strongly in the subgroup expressing HLA DR4 or DR1. This result suggests a link between antigen presentation by HLA DR4 or DR1 and expansion of V-β 13 T cell clones.

The potential involvement of pathogens suspected to trigger autism was investigated by examining T cell proliferation responses to peptide epitopes. As a group, the 24 autistic subjects did not show a decreased response to peptides derived from rubella virus, influenza A virus, herpes simplex virus type 1, cytomegalovirus, and *Clostridium tetani*. Another model of autism postulates that autism is induced by pathogens that possess epitopes identical to the hypervariable region 3 (HVR-3) of the HLA DR4 or DR1 alleles. Two antigens derived from the *Escherichia coli dna* J protein and the Epstein-Barr virus glycoprotein 110 peptides that contain sequences identical to the HVR-3 of the DR4 and DR1 alleles were examined for their ability to induce T cell proliferation in autistic and normal subjects. No effect of the DR4 or DR1 alleles on the response to these two antigens was detected. Therefore, both types of results do not support the model of immune tolerance in autism. However, average T cell proliferative activity was significantly lower in the same autistic subjects. This confirms many prior reports that reduced T-cell responses may shape susceptibility to autism.

Further understanding of how immune abnormalities and infectious agents lead to autism should guide development of preventive and therapeutic strategies for this disease.

(152 pages)

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Yong (Roger) Hu

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CHAPTER 1

INTRODUCTION AND PROJECT OUTLINE

AN OVERVIEW OF AUTISM

Autism, also called infantile autism or autistic disorder, is a developmental disorder of unknown etiology. Clinically, autism is characterized by obvious deficits in the ability to interact and communicate. The primary symptoms of autism are poor social development and communication, fixed imagination, and a limited scope of activities and interests (Gillberg, 1990). Most researchers now believe that autism is a multifactorial disorder (Gillberg and Coleman, 1992).

Autism is found throughout the world regardless of racial, ethnic, or social background. In the U.S., it occurs in 0.03-0.05% of the general population (American Psychiatric Association, 1994). There is a biased gender ratio in autism with 3-5 times as many males diagnosed with the disease (Rutter, 1978). Although autism affects males more frequently, symptoms in females are often more severe. Familial studies indicate that about 3% of the siblings of autistic subjects are also affected by autism, a 50-fold increase over the frequency seen in the general population (Folstein, 1985; Bolton and Rutter, 1990).

Like most developmental disorders, the symptoms of autism are age-sensitive. Autism is usually diagnosed when a child is 2 or 3 years of age (Rutter, 1978). During infancy, an affected child does not make eye contact, smile, or engage in other forms of communication with parents. In rare cases, the diagnosis of autism is made relatively late, when problems with social interactions and language development become evident.

Current behavioral treatments are designed to promote social involvement and improve communication skills (American Psychiatric Association, 1994). They usually consist of language training and practice, family counseling, and sometimes include the use of medication to control such symptoms as self-destructive behaviors, depression, or agitation. Even with these measures the majority of autistic individuals are severely impaired in adulthood. Only 1 in 50 patients with autism can work or live independently (Shaner, 1997).

CLINICAL FEATURES OF AUTISM

The diagnosis of autism is based entirely on behavioral traits. Correct diagnosis can be a challenge as autism is difficult to distinguish from other developmental disorders such as Rett Syndrome and Asperger Syndrome. Rett Syndrome affects only females and is apparent at a later age after normal early development (Gillberg and Coleman, 1992). Asperger Syndrome presents social deficits similar to those of autism, but affected individuals possess normal mental capacity and verbal and nonverbal communication skills (Gillberg and Coleman, 1992).

The standard criteria for diagnosis of autism are given in the Diagnostic and Statistical Manual of Mental Disorders of the American Psychiatric Association (1994). According to this manual, the three abnormalities of autism are: 1) dysfunctional social interactions and poor use of nonverbal communication; 2) poor use and understanding of spoken language; 3) programmed behaviors and limited imagination as manifested by rigid play formats and narrow interest scopes.

Minimal social participation or association is a striking feature of autism. Obvious

difficulties are seen using nonverbal communication through eye contact, facial expression, body posture, and gestures (Wing, 1980). Many autistic subjects also experience heightened sensory stimulation that affects their perceptions of and responses to their environment. As a consequence, autistic children react to stimuli in bewildering ways. Emotional aspects of the disease, such as unwarranted anger or happiness and fear of normally unprovocative stimuli, further inhibit normal social interactions.

Children with autism often have dramatic problems with verbal and nonverbal communication. Errors in pronunciation and grammar coupled with difficulties understanding verbal and body language are commonly observed (Bartak *et al.,* 1975). Some individuals have the ability to converse, but are impaired in their ability to initiate or continue conversations. Rejection of imitative social actions and use of stereotypical patterns of language are common characteristics of autism (Gillberg, 1990). These communication deficits further isolate autistic children.

The interests of autistic subjects are limited. Attention often is fixed upon a single object. Repetitive body movements and self-stimulating behavioral rituals, such as rocking or scratching, also are common manifestations of autism.

More than 75% of children with autism meet definitions of mental retardation, having an Intelligence Quotient (IQ) of < 70 (Lotter, 1966). About 40% of autistic children have an IQ of less than 40. Ironically, about 10% of autistic subjects exhibit extraordinary ability in one specific skill, such as memorization, mathematical calculation, musical expression, or art. Unfortunately, the same autistic child may be dysfunctional in other areas, unable to answer such basic questions as what did you eat for dinner? Who was yesterday's playmate?

Occasionally, other medical conditions are associated with autism. These include epilepsy (Taft and Cohen, 1971), vision and hearing impairments, fragile X syndrome (Steffenburg and Gillberg, 1986; Gillberg, 1992), encephalitis, chromosome translocation, maternal rubella, and tuberous sclerosis (Lotter, 1974).

BIOLOGY-BASED VIEWS OF AUTISM

Views of autism have evolved over the past 50 years from Kanner's early concept of autism as an outcome of poor parenting to more recent ideas of autism as a multifactorial, biologically based disorder (Gillberg, 1992; Burger and Warren, 1998). A synopsis of some of the major ideas of the etiology of autism is presented below. These hypotheses are summarized in Figure 1.1.

Psychogenic Theory

Kanner (1943), the first investigator to describe autism as a defined developmental disorder, believed autism resulted from parenting failure. He erroneously thought that autistic children came primarily from upper class families and that parents of autistic children failed to provide critical language training, social interactions, and physical attachment to their children because of their preoccupation with professional or social success. This idea came to be known as the psychogenic theory of autism.

Experimental evidence for Kanner's theory came from the pioneering studies of Harlow and McKinney (1971), who showed that rhesus monkey infants that were isolated from parents and all other social contacts developed symptoms resembling human infantile autism. Even when these animals were later released from isolation chambers and returned to their mothers, most continued to display autism-like symptoms of social

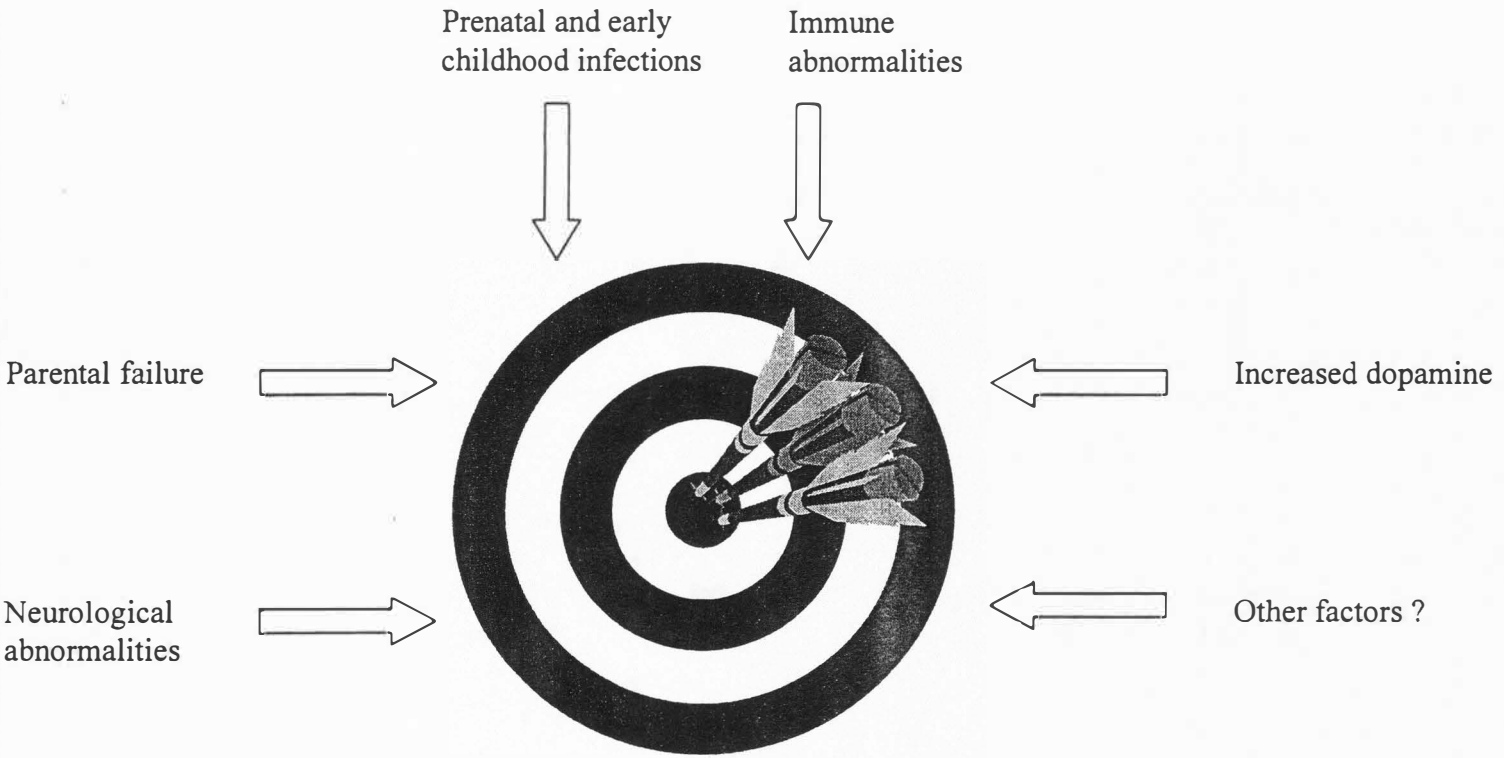

Fig. 1.1. Hypotheses for the etiology of autism.

isolation, poor communication, abnormal, repetitive movements, and self-damaging behavior.

Harlow and McKinney (1971) proposed that a lack of parental contact and social stimulation caused underdevelopment of the brain. They hypothesized that parental touch and emotional nourishment are essential to the normal development of the central nervous system in infant monkeys and humans. This psychogenic theory of autism became widely accepted.

Later studies revealed serious flaws in the psychogenic theory. A major discrepancy between theory and reality was the finding that the autism rates were independent of social class, economic status, patterns of feeding, language training, general sensory stimulation, and the degree of parental emotional attachment and nurturing (DeMyer *et al.,* 1972).

Genetic Factors in Autism

A role for genetic factors was suggested by a study of concordance in mono- and dizygotic twins (Folstein and Rutter, 1977; Folstein, 1985). The concordance of autism in monozygotic twins is about 36%, while the concordance in same-sex dizygotic twins approaches zero, arguing strongly for a role of genetic factors in the disease. The fact that concordance in monozyogotic twins is not perfect is also significant because it points to the existence of environmental factors in disease development. More recently, Warren and colleagues indicated that immunogenetic factors play a significant role in the development of autism (Warren *et al.,* 1986). Human leukocyte antigen (HLA) DR molecules present antigens to T effector cells and are essential for T cell function.

Particular HLA DR alleles were found at a much higher frequency in autism, suggesting that T cell functions are involved in the etiology of the disorder (Warren *et al.,* 1992, 1996).

Dopamine Imbalance

High levels of the central nervous system (CNS) neurotransmitter dopamine have been documented in a significant number of autistic individuals (Minderaa *et al.,* 1989; Barthelemy *et al.,* 1989). Dopamine regulates many CNS functions, including memory, hormone secretion, emotional status, and alertness. Dopamine levels in autistic individuals are elevated because the activity of dopamine hydroxylase, the enzyme that degrades dopamine, is reduced in the disease (Goldstein *et al.,* 1976; Lake *et al.,* 1977; Cohen *et al.,* 1977). Administration of dopamine analogs in some patients makes autistic symptoms more severe, while drugs that lower dopamine have the opposite effect (Campbell *et al.,* 1976, 1978). These findings clearly implicate dopamine imbalances in some autistic individuals.

Infectious Factors in Autism

Infections, especially viral infections, appear to be an important factor in autism. Rimland (1964) first found that children infected by rubella virus developed autism. Later, similar studies were reported by Desmond *et al.* (1967), Chess (1971), and Freedman *et al.* (1977). Taken together, these studies indicate a nearly 200-fold increase in the frequency of autism in rubella-infected children over that seen in the general population (Chess, 1977). Subsequently, other viruses have been implicated in autism through case studies. These include cytomegalovirus (CMV) (Stubbs, 1978), herpes

simplex virus 1 (HSV-1) (Ritvo *et al.,* 1990), varicella-zoster virus (VZV) (Knobloch and Pasaminick, 1975), and human immunodeficiency virus type 1 (HIV-1) (Schmitt *et al.,* 1991). Infection by these viruses during either prenatal or postnatal development is thought to be possibly involved in autism.

Immune system abnormalities and viral infections may interact in the etiology of autism. In this scenario, viral infections early in life induce B or T cell abnormalities that either cause autoimmunity or allow pathogen persistence. In either event, the consequence is CNS damage that may lead to autism. The studies reported in Chapter 2, 3 and 4 of this dissertation explore aspects of the potential interrelationship between infection and immune abnormalities in autism.

Immune Defects in Autism

Immune system defects appear to be an important factor in autism. Two broad classes of these defects have been investigated intensively during the past two decades: immune tolerance induced by early pathogen exposure and autoimmune responses directed against the CNS. The investigation of both forms of immune system abnormality forms a major part of this dissertation.

The hypothesis that immune tolerance induced by infection is a factor in autism came from the observation that a group of normal and autistic subjects revealed a striking difference in the ability to produce antibodies against rubella vaccine. Stubbs (1976) found that 6 of 15 of these autistic children did not produce antibodies against rubella vaccination challenge whereas all eight normal controls produced antibodies after rubella vaccination. Stubbs (1976) proposed that some cases of autism may result from immune

tolerance induced by prenatal or early postnatal rubella infection. This immune tolerance is postulated to lead either to persistence of the original pathogen or susceptibility to secondary infections. Persistent, low-level infection in immune-tolerant individuals may result in the CNS damage leading to autism (Stubbs, 1976).

The existence of CNS autoimmune diseases such as multiple sclerosis (Kennedy and Karpus, 1999) and Sydenham's chorea (Moore, 1996) prompted investigations of autoimmunity in autism. Autoimmunity directed against at least two CNS components has been discovered in autistic patients. Weizman *et al.* (1982) found T cell-mediated immune responses to human myelin basic protein in 13 of 17 autistic patients, but not in any of 11 patients suffering from other mental disorders. This report is important because it indicated that activated T cells are involved in autism and can target CNS structural proteins. Singh *et al.* (1993) independently found autoantibodies to myelin basic protein in children with autism but not in normal controls. Finally, Todd and Ciaranello (1985) discovered autoantibodies to CNS serotonin receptors in the cerebrospinal fluid of 7 of 15 autistic patients and in none of 15 normal controls. These studies provide compelling evidence for the existence of autoimmunity in autistic individuals. The fact that activated T cells easily cross the blood-brain barrier (Wekerle *et al.*, 1986) strengthens models of T cell-mediated autoimmune damage to the CNS system as a cause of autism.

Findings that indicated immune abnormalities in autism sparked a series of investigations into the cellular basis of these immune defects. These studies have uncovered an array of T cell alterations in autistic subjects. Stubbs *et al.* (1977) examined lymphocyte proliferation stimulated by phytohemagglutin (PHA) as an indicator of T cell function. PHA primarily stimulates CD4+ T cells. The lymphocytes of autistic subjects

displayed a significantly decreased proliferation response after stimulation with PHA (Stubbs, 1976). A similar investigation performed by Warren and his coworkers independently confirmed this finding (Warren *et al.,* 1986).

Warren's group went on to investigate the activity of natural killer (NK) cells in autism (Warren *et al.,* 1987). NK cell activity provides non-specific immune protection against viral infections and malignancy, and NK cells help to regulate immune responses. In many autoimmune disorders, including multiple sclerosis (MS), rheumatoid arthritis (RA), and Sjogren's syndrome (Sibbitt and Bankhurst, 1985; Takeda *et al.,* 1987), NK cell activity is decreased significantly. Warren *et al.* (1987) found that NK cell activity was reduced in 12 of 31 patients with autism and none of 23 normal individuals.

Two types of T cells are distinguished on the basis of their expression of the CD4+ or CD8+ cell surface marker proteins. CD4+ T cells, also known as helper T cells, work against intracellular antigens, and CD8+ T cells, also known as cytotoxic T cells, target extracellular antigens. Warren *et al.* (1986) reported that patients with autism had a reduced number of circulating CD4+ T cells, whereas numbers of CD8+ T cells were normal. This finding was confirmed by Yonk *et al.* (1990).

Activated T cells express surface proteins, called HLA DR molecules, which regulate T cell activation and T cell interactions. Warren *et al.* (1995) found that the DR+ marker was expressed in 14 of 26 (53%) autistic subjects compared to only 3 of 27 (11%) age-matched healthy controls. Additionally, comparisons between age-matched DR+ autistic and normal individuals showed an 8-fold increase in the number of DR+ T cells in autistic subjects. This indicates that T cell activation occurs in autism and suggests involvement of the immune system in the disease. A similar elevation of DR+ markers

has been reported in other autoimmune disorders (Effrench-Constant, 1994), including the autoimmune disease rheumatoid arthritis (Auger and Roudier, 1997).

The cytokine expression profiles of patients with autism are unusual and also suggestive of immune system dysfunction. Cytokines are major mediators of immune responsiveness. Singh and colleagues found elevated levels of interleukin type 2 (IL-2) (Singh *et al.,* 1991) and interleukin type 12 (IL-12) and interferon-γ (Singh, 1996) in the serum of autistic subjects. These results indicated the involvement of a class of helper T cells, known as Th1 cells. Gupta *et al.* (1998) also reported imbalances in cytokine levels in autistic individuals and increased levels of interleukin type 4 (IL-4) were found which is in contrast to the studies of Singh and his associates. These results suggest involvement of Th2 helper T cells in autism. Chapter 2 of this dissertation reexamines this issue of a role for Th1 or Th2 cells in autism using a more sensitive methodology.

Finally, there is evidence of non-T cell immune abnormalities in autism. In a search for abnormal B cell functions, Warren *et al.* (1997) uncovered lower serum levels of immunoglobulin A (IgA) in 20% of autistic subjects studied. IgA is the major immunoglobin secreted in the digestive tract, respiratory system, and milk. Warren and colleagues also found decreased levels of circulating complement type 4B (C4B) in some autistic patients (Warren *et al.,* 1995). C4B is an important element of the complement cascade and its downregulation would reduce the efficiency of antigen destruction.

TH1 VS TH2 CYTOKINES AND THEIR IMPLICATION IN HUMAN DISEASES

Blood is a heterogeneous mixture containing red and white blood cells. About 25% of white blood cells are lymphocytes, key components of the specific immune

system (Geha *et al.,* 1994). Among lymphocytes, B lymphocytes, or B cells, are responsible for humoral immunity, while T lymphocytes, also called T cells, control cellular immunity.

Th1 and Th2 Cytokines

There are distinct sets of T cells that perform different functions. CD8+ T cells, also known as cytotoxic T cells, defend against extracellular microorganisms. Activated cytotoxic T cells attack and kill these pathogens directly. CD4+ T cells, also called helper T (Th) cells, activate macrophages and B cells. There are two major types of CD4+ T cells, Th1 and Th2 (Romagnani, 1992, 1995, 1996, 1997). Th1 and Th2 cell types, their functions, and the cytokines they produce are shown in Figure 1.2. Th1 cells pick up cells that are infected with granular intracellular antigens. Eventually, macrophages are activated by Th1 T cells to engulf the infected cell. Th2 cells detect extracellular antigens, then activate B cells to produce specific antibodies. Th1 and Th2 CD4+ T cells produce distinct types of cytokines and these patterns of cytokine production are used experimentally to distinguish the T helper cell types (Mosmann and Coffman, 1989; Limaye *et al.,* 1990). Th1 cells primarily produce interferon-γ and interleukin type 2 (IL-2), whereas Th2 cells secrete IL-4 and IL-5 (Mosmann and Sad, 1996).

Role of Th1 and Th2 Cytokines in Disease

Helper T cell abnormalities play a role in disease. Activated Th1 cells are found in affected tissues of disease that include MS (Laman *et al.*, 1998; Windhagen *et al.*, 1998), RA (Miossec and van den Berg, 1997; Moore, 1999), autoimmune thyroid disease (Martin *et al.,* 1990), Crohn's disease (Raedler *et al.,* 1986), and acute allograft rejection

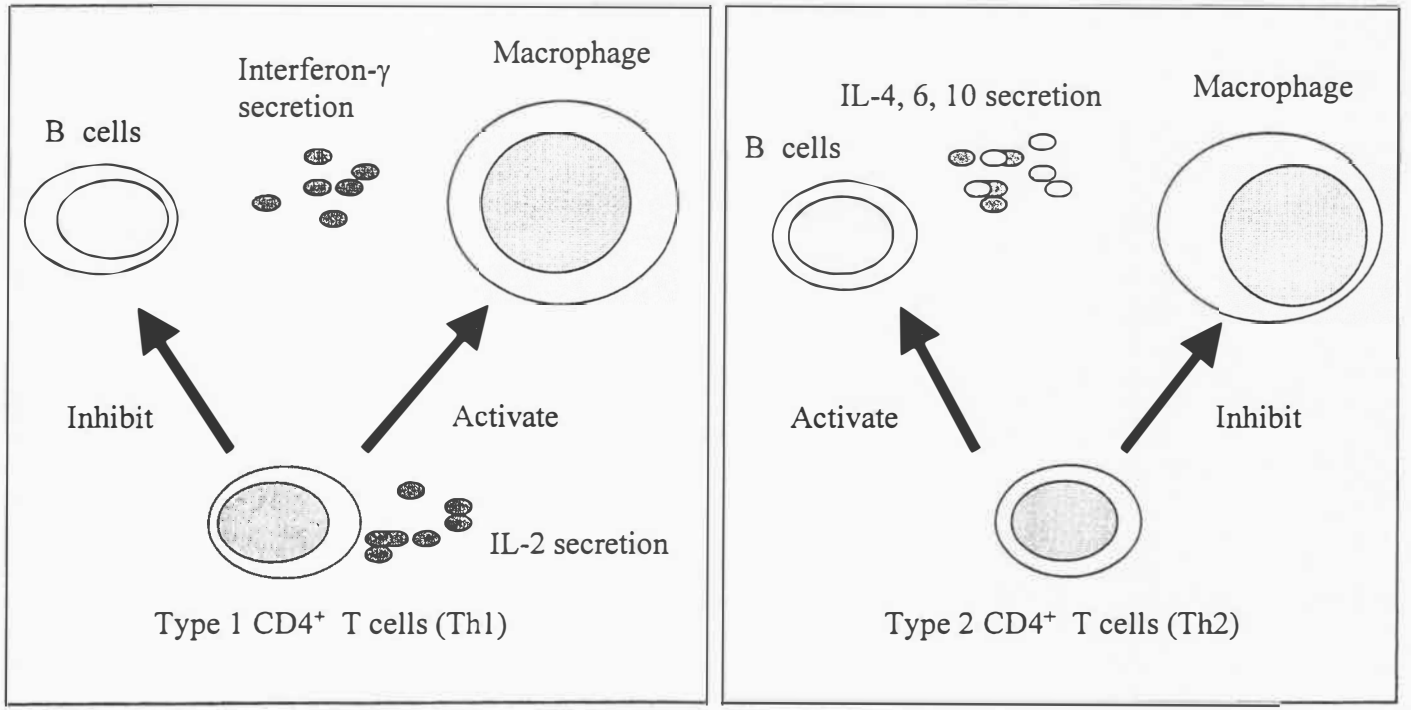

Fig. 1.2. Th1 and Th2 types of CD4+ helper T cells. Th1 helper T cells activate macrophages and inhibit B cells. Th2 helper T cells show the converse pattern, activating B cells and inhibiting macrophages. Activation of each T cell type is characterized by secretion of specific cytokines.

(De Carli *et al.,* 1994). In animal models, adaptive transfer of activated Th1 cells induces experimental autoimmune uveoretinitis and insulin-dependent diabetes (reviewed by Liblau *et al.,* 1995). Activated Th2 cells are present in tissues affected by progressive systemic sclerosis and chronic graft-versus-host rejection (Krenger and Ferrara, 1996). In all autoimmune diseases where helper T cells play a dominant role, cytokines in the peripheral blood accurately indicate the type of helper T cell that infiltrates affected tissues. For example, in animal models of multiple sclerosis, activated Th1 cells are found in the CNS (Link, 1998) and Th1-specific cytokines are elevated in the peripheral blood (Okuda *et al.,* 1998). Furthermore, in rheumatoid arthritis, distinct cytokine patterns correlate well with the disease stages (Kanik *et al.*, 1998).

Abnormal T helper cell activation may be involved in psychiatric disorders. Abnormal cytokine levels are detected in the cerebrospinal fluid of children suffering from obsessive disorder and schizophrenia (Mittleman *et al.,* 1997). In autism, Singh *et al.* discovered unusually high serum levels of IL-2 and IL-12 (Singh *et al.*, 1991; Singh 1996), indicating Th1 cell activation. Gupta *et al.* (1998) also found cytokine elevation in autism indicative of helper T cell activation, but saw an increase in IL-4, a marker of Th2 cell activation. The studies reported in Chapter 2 of this dissertation take an alternative experimental approach to reexamine the question of Th1 or Th2 cell activation in autism.

T CELL RECEPTOR REPERTOIRES IN HUMAN DISEASES

The T Cell Receptor (TCR)

T cell receptors are transmembrane proteins that allow antigen recognition. The

predominate TCR is a heterodimer made of α and β chains, although TCRs composed of γ and δ chains occur at lower frequency. The structure of the αβ TCR heterodimer is presented in Figure 1.3. Each T cell expresses only a single type of TCR heterodimer and as a consequence recognizes a restricted set of antigens presented in the context of major histocompatibilty complex (MHC) molecules present on the surface of antigen-presenting cells (Geha *et al.,* 1994). Binding of the antigen-MHC complex to the TCR activates or inhibits T cell proliferation. Currently, 50 α and 60 β TCR chains are known (Spinella and Robertson, 1994; Garcia *et al.*, 1999). These allow formation of a huge number of heterodimers that can recognize an even larger number of antigens (Imberti *et al.*, 1993; Arden *et al.*, 1995; Vandenbark *et al.*, 1996). Each polypeptide chain is composed of a constant and a variable domain. The specificity of T cell-antigen interaction is due primarily to the type of β chain that is expressed (Geha *et al.,* 1994).

The TCR repertoire is determined by many factors, including genetic background. Expansion of specific T cell clones is observed in many disease conditions, including T cell-mediated autoimmune disorders, during infection (Islam *et al.*, 1996) and stimulation by superantigens. Expanded T cell clones are identified on the basis of the T cell receptors they express, with the specific type of variable region of the β (V-β) chain serving as a marker. Two methods are available for detection of specific V-β chains: flow cytometry and reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR has the advantages of greater sensitivity and the ability to detect all major specific V-β chains TCR, and is now the method of choice.

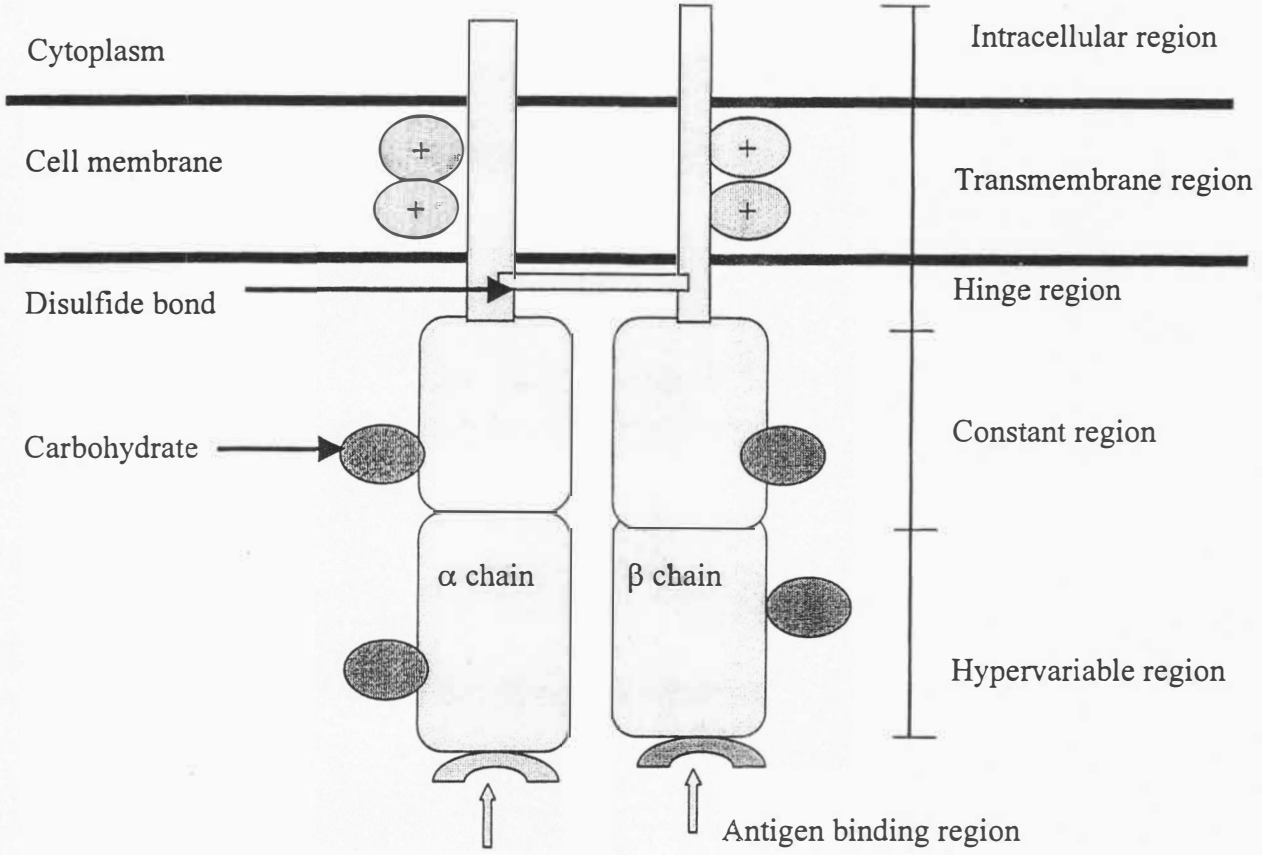

Fig. 1.3. T cell receptor (TCR) structure.

Altered TCR Repertoires in Disease

T cell clone expansion is a hallmark of autoimmune diseases. These documented autoimmune diseases include MS (Wucherpfennig and Hafler, 1995; Lozeron *et al.*, 1998), type I diabetes (Atlan-Gepner *et al.*, 1997), systemic lupus erythematosus (Furukawa *et al.*, 1996; Holbrook *et al.*, 1996; Mato *et al.*, 1997), sarcoidosis (Moller *et al.*, 1988), Kawasaki disease (Abe *et al.*, 1993), microscopic polyarteritis (Simpson *et al.*, 1995), primary biliary cirrhosis, Sjogren's syndrome, and RA (Jenkins *et al.*, 1993). In every one of these disorders, one or a few helper T cell clones form the predominant activated T cell population. For example, T helper cells are known to infiltrate affected nervous tissue of MS patients and RT-PCR has shown that these cells express V-β 8 almost exclusively. In RA, similar RT-PCR analysis shows that either V-β 14 or V-β 17 is expressed by the helper T cells collected from synovial fluid of affected joints. The same V-β regions are expressed by the majority of helper T cells in peripheral blood of RA patients, indicating a good correlation between expanded T cells populations in peripheral blood and affected tissue. MS and RA provide two examples of autoimmune diseases in which a specific T cell population is expanded. The studies reported in Chapter 3 of this dissertation use RT-PCR of RNA isolated from peripheral blood to learn if particular T cell clones are expanded in autism.

In animal models of autoimmune disorders, there is good evidence that T cell clone expansion is a cause rather than a consequence of disease. Transfers of splenic T cells from animals with experimentally induced type I diabetes or RA results in the development of the corresponding diseases in genetically compatible animals. In murine

experimental autoimmune thyroiditis, the unique and causative TCR V-β clones are also identified (McMurray *et al.*, 1996).

The enhanced expression of specific cytokines and T cell clone expansion may be related (Parronchi *et al.*, 1991; Olsson *et al.*, 1995). Initial stimulation of T cell clones by exposure to antigens will induce specific patterns of cytokine secretion. In turn, these cytokines feed back upon the initially stimulated T cell clone in an autocrine feedback loop to further stimulate clonal expansion. This clonal expansion fueled by increased cytokine expression may lead to autoimmune diseases. This model is supported by investigations of MS where it was found that upregulation of Th1 cytokine expression and T cells clones expressing V-β 8 were interrelated in both MS patients and in animal models of the disease (Olsson *et al.*, 1995)

In addition to serving as a marker, the V-β region expressed by T cell clones presents a target for therapy. In animal models of MS, administration of monoclonal antibodies against V-β 8 decreased the severity of symptoms (Urban *et al.,* 1988; Vandenbark *et al.,* 1989; Acha-Orbea *et al.*, 1988). Knowledge of potential T cell clones that predominate in autism may offer the hope for similar therapy.

Susceptibility to most autoimmune diseases involves a significant genetic component. An important genetic marker for susceptibility to any autoimmune disease is the MHC genotype (Albani *et al.*, 1992; Ikeda *et al.*, 1996), especially alleles of the DR locus. This locus encodes DR proteins that are key elements of the MHC that presents antigens to T cells (Geha *et al.,* 1994; Tuokko *et al.*, 1998). Particular DR alleles occur at high frequency in many autoimmune disorders (Geha *et al.,* 1994; Tuokko *et al.*, 1998).

For example, in MS DR2 is commonly present, in rheumatoid arthritis DR4 predominates, in type I diabetes DR3 and DR4 occur frequently, and in myasthenia gravis, DR3 is frequently present. A very high frequency of DR4 or DR1 is associated with autism (Warren *et al.*, 1995). The reasons behind the association between specific DR alleles and autoimmune diseases are unknown, but one hypothesis is that particular DR alleles present antigens derived from infectious pathogens in a manner that induces T cells to target not only the foreign antigen but self-antigens as well (Ou *et al.*, 1998, 1999; Williams *et al.,* 1993). This leads to expansion of self-reactive T cell populations that destroy host tissue.

INFECTIOUS AGENTS SUSPECTED IN AUTISM

Epidemiological investigations and case studies both suggest a close link between viral infections early in life and autism. These viruses include rubella (Rimland, 1964), CMV (Stubbs, 1978), HSV-1 (Ritvo *et al.*, 1990), VZV (Knobloch and Pasaminick, 1975), mumps (Deykin and Macmahon, 1979), and HIV-1 (Schmitt *et al.,* 1991). Rubella is the strongest candidate pathogen in autism with other viruses implicated primarily through case studies. The evidence for rubella virus, CMV, and HSV-1 as causative agents of autism is discussed below.

Rubella Virus

After a rubella virus epidemic occurred in New York City, 243 infected children were followed in a prospective study. Within this group 10 subjects developed autism with full symptoms and 8 subjects with partial symptoms (Rimland, 1964). The infected

children were followed, and another 4 subjects were later diagnosed with autism (Chess *et al.*, 1978). This is a remarkably high frequency of autism, roughly 175-fold greater than that of the general population. In another study of 210 rubella virus-infected children, 7% were diagnosed with autism (Stubbs *et al.,* 1977). Small-scale investigations in Italy (Elia *et al.,* 1990) and Utah (Ritvo *et al.,* 1990) uncovered the same association between childhood rubella virus infection and autism. An important point stemming from these studies is that autism may develop either shortly (Freedman *et al.*, 1977) or a significant time after acute rubella virus infection (Pfefferbaum, 1996).

Rubella virus is best known for causing birth defects when infection occurs during pregnancy (congenital rubella syndrome), especially during the first trimester. In congenital rubella syndrome, rubella virus particles present in the maternal circulation cross the placenta and damage the fetus (Lim *et al.,* 1995; Lane *et al.,* 1996). Rubella virus infection also causes encephalitis that leads to cognitive, motor, and behavioral deficits that are first seen in early infancy. Rubella-induced encephalitis is long lasting, with infectious particles present in blood and cerebrospinal fluid 1 year after birth in 80% of congenitally infected infants (Cradock-Watson *et al.,* 1989; Katow, 1998). *In vitro* studies also indicate that rubella virus attaches to human lymphocytes and substantially decreases lymphocyte functions (Plotkin *et al.*, 1965). Although the role of rubella infections in autism is unknown, both CNS damage and alteration of lymphocytes activity are two possibilities.

CMV

Congenital CMV infection is a significant health problem in the United States

(Starr *et al.,* 1970). The view that CMV infection is a factor in autism comes from a series of case studies. Stubbs (1978) reported a case of an autistic patient who suffered congenital CMV infection. He speculated that congenital CMV infection could have led to the autistic symptoms probably through the same mechanisms that links rubella virus infection and autism. In a prospective study of 72 infants congenitally infected by CMV and followed for 3 years (Blattner, 1974), three cases of autism developed. In other studies, maternal CMV infection during pregnancy was suspected to produce autism. In these cases, congenital CMV infection of autistic children born to CMV-infected mothers was indicated by the presence of antibodies to CMV, viral particles in urine, hearing loss, and damage to the retina that appeared to be the result of CMV infection (Markowitz, 1983; Stubbs *et al.,* 1984; Ivarsson *et al.,* 1990).

HSV-1

The case for HSV-1 in autism comes from case studies showing antibodies against HSV-1 in autistic children and their mothers and an association between HSV-1-induced encephalopathy and autism. Like rubella virus and CMV, neonatal HSV-1 infection occurs commonly and affects about 5 per 10,000 of newborn infants in the U.S (Brown *et al.,* 1991). Delong and his associates were the first to report a possible link between congenital HSV-1 infection and autism (Delong *et al.,* 1981). In their study, three boys with autism were found to have had congenital HSV-1 infection that led to encephalopathy. These three individuals were asymptomatic for autism prior to their HSV-1-induced encephalopathies. Neurological examinations revealed CNS damage in two of these autistic children. In one case, damage to the left temporal lobe was found

and a high serum antibody titer for HSV-1 was detected. In another case, autism developed in a previously normal child 10 days after developing HSV-induced encephalitis (Gillberg, 1986). Bilateral damage in the temporal lobes of the second patient, presumably caused by HSV-1 infection, was also detected (Gillberg, 1986). Finally, Ritvo *et al.* (1990) found two cases in which autistic children and their mothers had antibodies against HSV-1 and speculated that there may be a causal relationship.

Vaccination

An abnormal response to vaccination has been suggested to be an important factor in the etiology of autism. There are anecdotal reports of children developing autism within months of immunization with measles-mumps-rubella (MMR) or diptheria-pertussis-tetanus (DPT) vaccines. Munyer *et al.* (1975) found that long-lasting, depressed lymphocyte functions developed in some individuals after MMR vaccination, and Rossier *et al.* (1994) reported an absence of cell-mediated immunity to rubella virus in 19 of 25 normal children 5 years after MMR vaccination. The generally depressed immune function seen in autistic patients (Yonk *et al.,* 1990; Warren *et al.,* 1990) and these observations of depressed immune functions following vaccination have led to the hypothesis that some autistic individuals develop their symptoms as a consequence of vaccine-induced immune depression (Campbell, 1989; McFarland, 1999). Possibly the decreased immune functions induced in some individuals allow persistent secondary viral infections that damage the CNS and lead to autism.

RESEARCH GOALS

This dissertation examined three questions related to immune abnormalities in the

etiology of autism. The first question was which type of helper T cell, Th1 or Th2, is involved in the disease. Two previous works showed expansion of helper T cell populations in autistic individuals, but came to conflicting conclusions regarding which helper T cell type was involved (Gupta *et al.,* 1998; Singh, 1996). The second question was whether a particular T cell clone predominates in autistic individuals. This would provide further evidence of altered T cell immune functions in autism. The final question of this study asked if specific pathogens are involved in autism.

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CHAPTER 2

CYTOKINE EXPRESSION PATTERNS INDICATE A Th1 TYPE OF T CELL RESPONSE IN AUTISM

ABSTRACT

Abnormal helper T cell function may underlie the pathology of autoimmune disorders and some psychiatric diseases of children, including autism. Helper T cells activate and inhibit a variety of other effector cells and are classified as Th1 or Th2 types based on the effector cells they control. Th1 cells secrete interleukin type 2 (IL-2), interferon-γ and interleukin type 12 (IL-12), and Th2 cells secrete interleukin type 4 (IL-4). Two previous reports have shown that helper T cells are involved in autism, but came to conflicting conclusions regarding whether these were Th1 or Th2 cells. In order to clarify which type of helper T cell is associated with autism, we employed competitive reverse transcription-based polymerase chain reaction (RT-PCR) to examine the profile of cytokines expressed in autistic and normal individuals. The expression of interferon-γ, IL-2, and IL-4 mRNA was examined in phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) obtained from 24 autistic subjects and 19 normal controls. Levels of interferon-γ and IL-2 mRNA were significantly higher ($p < 0.05$ and $p < 0.0002$, respectively) in the autistic group. IL-4 was detected in only 1 of 24 autistic subjects and in 5 of 19 of normal individuals ($p < 0.005$). These results indicate that activated Th1 T cells are associated with autism.

INTRODUCTION

Autism, also called infantile autism or autistic disorder, is a major pervasive developmental disease with an occurrence of 0.03-0.05% in the general population (Shaner, 1997). It is characterized by social isolation, language deficits, and narrow activity and interest scopes. The etiology of autism is unknown, and multiple causes have been postulated, including genetic factors (Folstein and Rutter, 1977; Folstein 1985), neurological damage (DeMyer *et al.*, 1972), immune dysfunction (Warren *et al.*, 1986, 1987; Todd *et al.*, 1988; Singh *et al.*, 1993), and viral infections (Chess, 1977; Matrkowitz, 1983). Immune abnormalities, especially T cell-mediated dysfunction (Yonk *et al.*, 1990), have been intensively investigated in autism. Abnormal T cell functions (Stubbs *et al.*, 1977; Weizman *et al.*, 1982), T cell number (Warren *et al.*, 1986, 1990), and the helper/depression T cell ratio (Plioplys *et al.*, 1994; Warren *et al.*, 1995) are all altered in autistic subjects.

CD4+ T cells, also known as helper T cells (Th), are at the core of T cell-mediated immunity. There are two distinct subpopulations of CD4+ T cells (reviewed by Olsson, 1995). Th1 cells activate macrophages, and inhibit B cells and are responsible for acute rejection during organ transplantation and for elimination of intracellular pathogens. Th2 cells activate B cells, and inhibit macrophages and cause antibody-related autoimmune reactions. CD4+ T cells control effector cells largely through the cytokines they secrete. The predominant cytokines secreted by Th1 cells are interferon-γ and IL-2 and the predominant cytokines secreted by Th2 cells are IL-4, interleukin type 5 (IL-5), and interleukin type 10 (IL-10) (Mosmann and Sad, 1996). CD4+ T cell activation can lead to autoimmune diseases in human and animal models. Th1 cell activation is also

responsible for organ-specific autoimmune diseases that include experimental autoimmune uveoretinitis and insulin-dependent diabetes (reviewed in Liblau *et al.*, 1995), and Th1-specific cytokines such as IL-2 and interferon-γ are detected in autoimmune thyroiditis, type I insulin dependent diabetes mellitus, multiple sclerosis, peptic ulcers, and Crohn's disease (reviewed by Romagnani, 1995, 1996, 1997; Adorini and Sinigalia, 1997). In animal models, injection of activated Th1 cells into naive animals generates experimental autoimmune encephalomyelitis (EAE) (reviewed in Fabry *et al.*, 1994). Altered levels of Th1- or Th2-specific cytokines have also been observed in childhood obsessive-compulsive disorder and schizophrenia, suggesting an involvement of CD4+ T cells in these psychiatric diseases (Mittleman *et al.*, 1997).

The potential involvement of CD4+ T cells in autism has been investigated by examining cytokine expression patterns either in serum or in PBMC. Singh and his coworkers (Singh *et al.*, 1991; Singh, 1996) reported that the Th1-specific cytokines IL-2, IL-12, and interferon-γ were elevated in the serum of autistic individuals, whereas Gupta *et al.* (1998) reported that IL-4, a Th2-specific cytokine, was elevated in PBMC of autistic subjects. In this paper, we reexamined the issue of which type of CD4+ T cell, Th1 or Th2, is involved in autism by using RT-PCR to measure IL-2, interferon-γ, and IL-4 mRNAs present in PBMC.

RT-PCR was chosen to assay cytokine expression because of its high sensitivity. This technique has been widely utilized for studies of cytokine expression in human autoimmune disorders (Di Fabio *et al.*, 1994; Bost *et al.*, 1995; Than *et al.*, 1997). An assumption of the method is that mRNA levels are a reflection of active cytokine. In cases where this assumption has been examined, RNA levels have been shown to be a

good predictor of cytokine levels. For example, in a study of IL-10 expression in different human T cell lines, mRNA levels detected by RT-PCR were well correlated with IL-10 protein in serum, blood, and ascitic fluid as measured by enzyme-linked immunosorbent assay (ELISA) (Bost *et al.*, 1995).

The results reported here indicate that the Th1-specific cytokines IL-2 and interferon-γ were elevated in autistic subjects and that the Th2-specific cytokine IL-4 was produced at detectable levels in only a single autistic individual. These results indicate that Th1 cells are activated in autism.

MATERIALS AND METHODS

Study Group

This study included 24 autistic (22 males and 2 females) and 19 normal subjects (15 males and 4 females) (Table 2.1). Ages ranged from 2-19 years in the autistic group and from 7-16 years in the normal group. Diagnosis of autism was made by child psychiatrists according to parameters of American Psychiatric Association (1994).

Isolation of Total RNA

Blood was collected in heparin-coated tubes and peripheral blood mononuclear cells (PBMC) were isolated by histopaque centrifugation. The cells were washed with RPMI-1640, resuspended in 10% dimethyl sulfoxide and 90% fetal bovine serum (FBS), and cryopreserved at –80°C. For analysis, PBMC were thawed and cultured in RPMI-1640 supplemented with 10% FBS and 10 μg/ml PHA (Gibco, BRL) for 5 days at 37°C in a humidified, 5% CO_2 atmosphere. The PBMC were harvested and collected by

Table 2.1
Sex and Age of Subjects

Autistic Subject	Sex	Age	Normal Subject	Sex	Age
#1	M	2	#1	M	7
#2	M	2	#2	M	9
#3	M	3	#3	M	9
#4	F	4	#4	M	10
#5	F	5	#5	M	10
#6	M	5	#6	F	11
#7	M	5	#7	M	11
#8	M	6	#8	M	11
#9	M	7	#9	F	11
#10	M	7	#10	M	12
#11	M	7	#11	M	12
#12	M	7	#12	M	13
#13	M	8	#13	F	13
#14	M	8	#14	M	14
#15	M	9	#15	M	14
#16	M	11	#16	M	14
#17	M	11	#17	M	15
#18	M	11	#18	F	15
#19	M	12	#19	M	16
#20	M	13			
#21	M	13			
#22	M	14			
#23	M	16			
#24	M	19			

centrifugation and RNA was extracted from the pelleted cells using the Trizol RNA extraction kit according to the manufacturer's directions (Gibco, Gaithersburg, MD).

Quantification of Cytokine mRNAs

cDNA was synthesized by random hexamer priming with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) using a kit according to manufacturer's directions (Boehringer Mannheim, GmbH, Germany). To minimize mRNA degradation, RNAase inhibitor at a final concentration of 1 unit/μl was added to the cDNA synthesis reaction.

Interferon-γ and IL-4 mRNA levels were determined by RT-PCR using commercial kits purchased from Clontech (Palo Alto, CA). These kits use specific "mimic" cDNAs added at known concentration to the reaction. The mimic cDNAs are cloned versions of the interferon-γ and IL-4 sequences that contain a small insertion to distinguish their amplification products from that of the endogenous sequences. These mimics and the cDNA synthesized from endogenous mRNA compete for the same primer set and other components of the PCR system. The sequences of oligonucleotide primers used in this study are given in Table 2.2. Precautions to avoid contamination in the PCR included the use of aerosol barrier tips and RNase inhibitor treatment of pipets, glassware, and bench surfaces. Negative controls lacking template DNA were included in all PCR analyses. In no case was there evidence of spurious amplification.

For competitive RT-PCR of interferon-γ and IL-4, 2 μl of cDNA synthesized from PBMC mRNA was placed into five tubes. Each tube contained a primer pair and 2 μl of a 1:3 dilution series of mimic cDNA with a starting amount 5.1×10^7 molecules of

Table 2.2
Oligonuleotide Primers Used in the Cytokine Study

Cytokine	Sequences	Size	
		PCR	Mimic
Interferon-γ 5'	5'-GCATCGTTTTGGGTTCTCTTGGCTGTTACTGC-3'	427 bp	570 bp
Interferon-γ 3'	5'-CTCCTTTTTCGCTTCCCTGTTTTAGCTGCTGG-3'		
IL-4 5'	5'-CGGCAACTTTGACCACGGACACAAGTGCGATA-3'	344 bp	504 bp
IL-4 3'	5'-ACGTACTCTGGTTGGCTTCCTTCACAGGACAG-3'		
IL-2 5'	5'-AACTCCTGTCTTGCATTGCA-3'	441 bp	
IL-2 3'	5'-GTGTTGAGATGATGCTTTGAC-3'		
Actin 5'	5'-ATGTTTGAGACCTTCAACAC-3'	489 bp	
Actin 3'	5'-CACGTCACACTTCATGATGG-3'		

interferon-γ and 5.6×10^6 molecules of IL-4. Mimic cDNAs and primers were purchased from Clonetech (Palo Alto, CA). IL-2 mRNA levels were measured relative to actin mRNA according the method of Haraguchi *et al.* (1995). Actin mRNA expression is assumed to be constitutive. Separate PCRs were performed to amplify IL-2 and actin cDNA, but analyzed in the same condition.

PCR was performed in a 15-μl mixture containing 10 mM Tris (pH 8.3), 50 mM KCl, 1.2 mM $MgCl_2$, 1 mM dNTP, 1 unit Taq DNA polymerase (Fisher Scientific, Pittsburgh, PA) and 45 pmol of each primer pair. PCR was carried out with an initial 5-minute denaturation at 94°C followed by 30 cycles of the following profile: 1 minute at 94°C, 1 minute at 64°C, and 2 minutes at 72°C. Pilot experiments were performed to establish conditions under which the PCR remained in exponential phase. Four μl of each PCR product was analyzed by electrophoresis through 1.5 % agarose gels. Gels were stained with ethidium bromide, visualized under UV light, and photographed with Polaroid 667 film.

The levels of interferon-γ and IL-4 mRNA were determined by comparison between the endogenous and mimic cDNA amplification products. Five different PCRs in which each PCR containing a different dilution of the mimic cDNA were run for each cytokine from each subject. Whenever the densities of the mimic and endogenous cDNA products matched, this indicated that the starting target cDNA concentrations were equal to that of their corresponding mimic cDNA. Tests of this method using a fixed and known amount of endogenous cDNA and varying amounts of mimic cDNA verified the accuracy of this method (data not shown). Relative levels of IL-2 were determined by

comparison of the intensity of the amplification products of the IL-2 and actin mRNAs from the same individual. This ratio was termed arbitrary optical density units (AODU). The photograph of the stained agarose gel was scanned and the intensities of the bands were determined using a densitometer (Biosoft Image System, Ferguson, MO). Mean values for each cytokine present in the autistic and normal groups were calculated and evaluated for statistically significant differences using Student's t test.

The levels of Th1- and Th2-specific cytokine mRNAs in the autistic and normal groups were compared in two different ways. In the first method, cytokine levels were averaged for the autistic and normal groups and the significance of these differences was analyzed using Student's t test. The second method compared the number of individuals in each group that expressed a particular cytokine to any detectable level and the significance of intergroup differences was assessed by the χ^2 test. These independent methods of analysis provide a measure of confidence regarding differences between autistic and normal individuals.

RESULTS

Levels of the Th1-specific cytokines interferon-γ and IL-2 are shown as scatter plots in Figures 2.1 and 2.2, respectively, and are given in Table 2.3. Because interferon-γ mRNA levels varied widely in both groups, they are shown as $\log_{10}$-transformed values in Figure 2.1. The mean level of interferon-γ expression was 2.5-fold higher in the autistic group and this difference from the normal group was significant ($p < 0.05$). As for interferon-γ, there also is considerable variability in IL-2 expression levels in both groups. The autistic group expressed an average 3-fold higher level of IL-2 mRNA

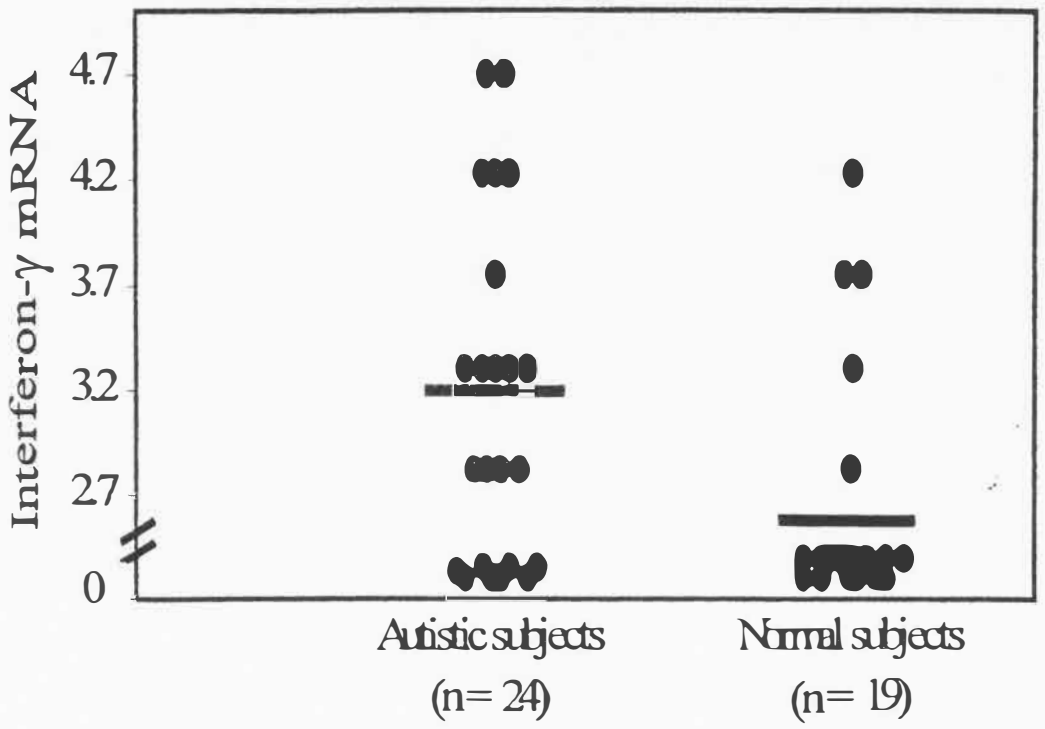

Fig. 2.1. Interferon-γ mRNA expression in autistic and control groups. The $\log_{10}$-transformation of interferon-γ mRNA molecules per μg of total RNA is plotted for each autistic and normal subject. Any value below the break mark (//) indicates undetectable levels of expression. The black horizontal bars (-) show the mean of the group, and the sample size in each group is given by the n value.

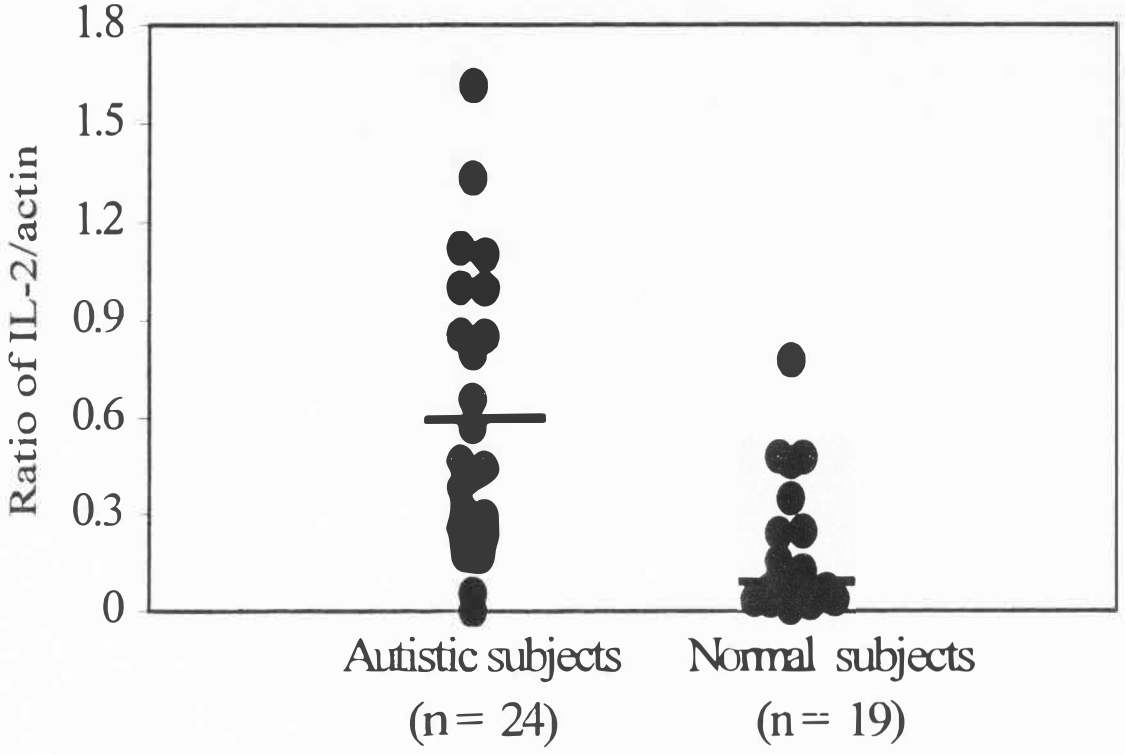

Fig. 2.2. Relative IL-2 mRNA expression in autistic and control groups. RT-PCR was performed as described in Methods. IL-2 mRNA expression is given as the ratio of the IL-2 to actin PCR products in the same subject. The black horizontal bars (-) show the mean of the group, and the sample size in each group is given by the n value.

Table 2.3

Cytokine Expression in Autistic and Normal Subjects

	Interferon-γ		IL-2		IL-4	
Group	Autistic	Normal	Autistic	Normal	Autistic	Normal
No. Detectable	15/24	5/19	22/24	8/19	1/24	5/19
% detectable	63%	26%	92%	42%	4%	26%
χ^2 test	$p < 0.025$		$p < 0.005$		$p < 0.005$	
$\mu \pm SD$[a]	12400 ± 7900[b]	4500 ± 8200[b]	0.60 ± 0.43[c]	0.18 ± 0.20[c]	240 ± 112[b]	1300 ± 1960[b]
Student's t test	$p < 0.05$		$p < 0.0002$		NA[d]	

[a]Mean ± standard deviation.
[b]Cytokine molecules/μg total RNA isolated from PBMC.
[c]The ratio between IL-2 and actin mRNA expression.
[d]Not applicable.

than did the normal controls ($p < 0.0002$). The expression of both of major Th1-specific cytokines in the autistic group indicates that Th1 CD4+ T cells are activated in many autistic individuals.

Levels of the Th2-specific cytokine IL-4 mRNA are shown as a scatter plot in Figure 2.3 and are given in Table 2.3. Only one of the autistic subjects and 5 of the normal subjects expressed detectable levels of IL-4 mRNA. Because of the low numbers of individuals expressing IL-4 in each group, Student's t test could not be used to analyze these results.

As shown in Table 2.3, the numbers of individuals in each group who expressed particular cytokines are also displayed. Interferon-γ was detected in 15 of 24 (62.5%) autistic subjects and 5 of 19 (26.3%) control subjects. This difference was significant ($p < 0.025$ by χ^2). IL-2 was detected in 22 of 24 (91.7%) autistic subjects and 8 of 19 (42.1%) normal controls. This difference was also significant ($p < 0.005$ by χ^2). In contrast to the Th1-specific cytokines, a higher number of control subjects expressed the Th2-specific cytokine IL-4 than did autistic subjects. Five of 19 (26%) control subjects and 1 of 24 (4%) autistic subjects expressed IL-4. This difference also was significant ($p < 0.005$ by χ^2).

The mean age for the autistic and normal study groups was 8.5 and 11.9 years, respectively. Although Student's t test evaluation of this age difference indicated it was not significant, the possibility that the elevated cytokine expression in the autistic group was due to the younger average age of these individuals was examined. The levels of IL-2 and interferon-γ were plotted against subject age and are show shown in Figure 2.4.

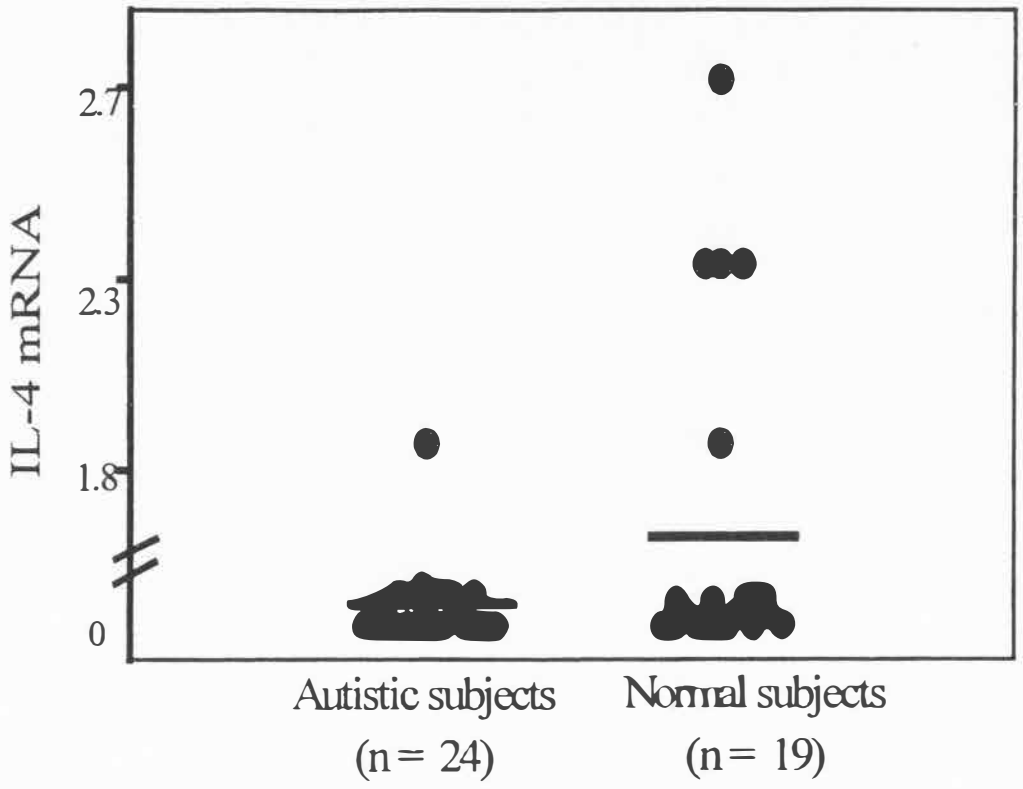

Fig. 2.3. IL-4 mRNA expression in autistic and control groups. The $\log_{10}$-transformation of IL-4 mRNA molecules per μg of total RNA is plotted for each autistic and normal subject. Any value below the break mark (//) indicates undetectable levels of expression. The black horizontal bars (-) show the mean of the group, and the sample size of each group is given by the n value.

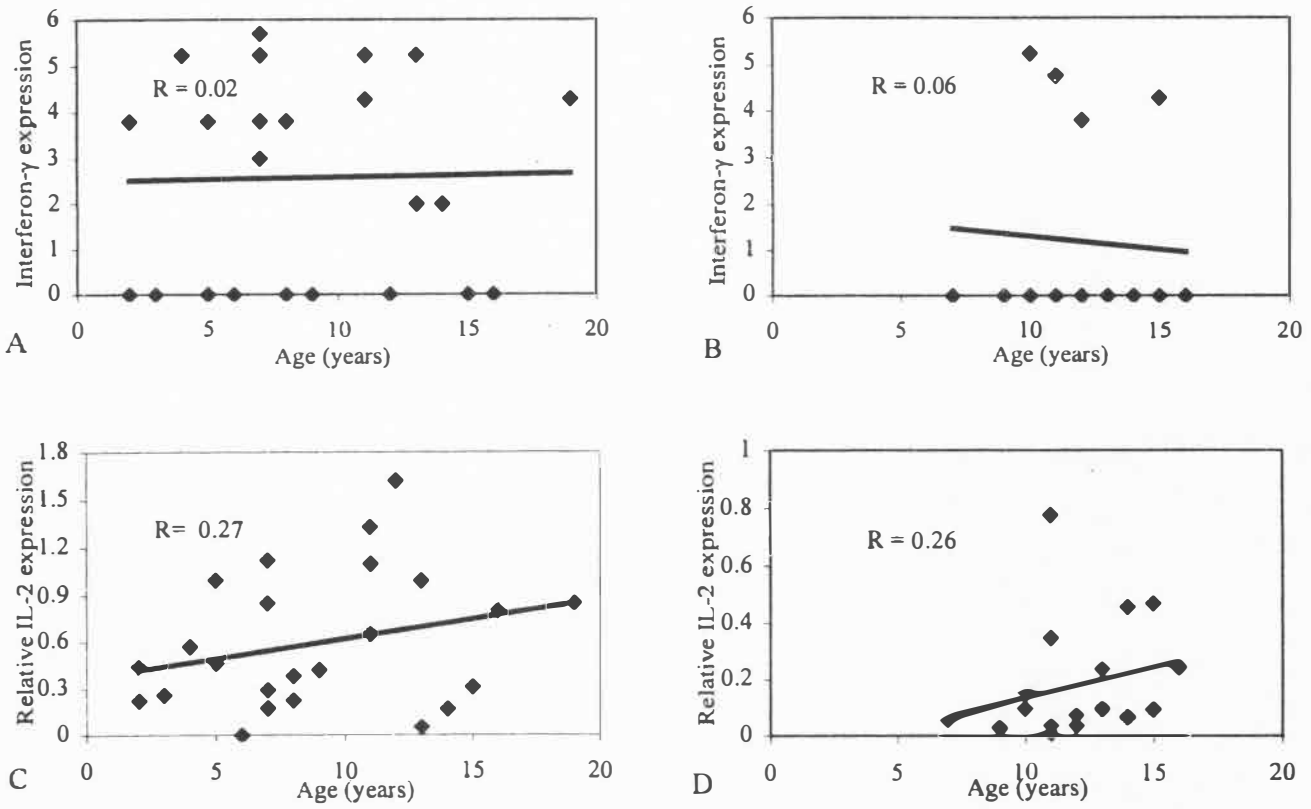

Fig. 2.4. The relationship between age and cytokine expression in autistic and normal individuals. Graph panels A and B show the $\log_{10}$-transformation of interferon-γ mRNA molecules per μg of total RNA in autistic (A) and normal (B) individuals. Graph panels C and D show IL-4 expression relative to that of actin in autistic (C) and normal (D) individuals. The corresponding r values are given in each panel.

There was no significant correlation between cytokine expression and age (r values ranged between – 0.06 and + 0.27). These results indicate that the difference in cytokine expression between groups was not age-related. The low frequency of individuals expressing IL-4 made age-specific comparisons meaningless in these study groups.

Since there were more females in the normal control group than in the autistic group, the possibility of a gender-specific difference in cytokine expression was examined by comparing the expression of IL-2 and interferon-γ in males and females within each study group and in the two groups as a whole. Student's t test indicated that gender played no role in the expression of these cytokines (data not shown). The low frequency of individuals expressing IL-4 made gender-specific comparisons meaningless in these study groups.

DISCUSSION

The current study supports an association between autism and increased expression of Th1-specific cytokines. A significant elevation of interferon-γ and IL-2 mRNA levels and a higher frequency of individuals expressing interferon-γ and IL-2 mRNAs was found in this study group of 24 autistic subjects. This indicates that there is a Th1 type of immune response in many autistic individuals. At the same time, there was almost no expression of IL-4 in the autistic group. This result argues against a Th2 type of response in the autism.

The results reported here agree with conclusions drawn by Singh and associates who used an ELISA-based method of cytokine analysis and found increased levels of IL-2 and IL-12 in autistic individuals (Singh *et al.*, 1991; Singh, 1996). Like IL-2, IL-12 is

an indicator of a Th1 response. Our and Singh's results contrast with those of Gupta *et al.* (1998), who reported a Th2 response in autistic individuals. Differences exist between all these studies and include: 1) Gupta and associates' use of fresh rather than cryopreserved PBMC; 2) the younger age of study subjects investigated by Gupta and associates; and 3) the use of different methods (RT-PCR, ELISA and flow cytometry) to assess cytokine production. It is unlikely that the use of cryopreserved rather than fresh cells account for the different results because cryopreservation of PBMC has been reported not to affect cell activation markers, viability, T cell function, preferential stimulation of CD4+ vs. CD8+ T cells (van Lunzen *et al.*, 1995), or the repertoire of T cell receptors β chains (see Appendix A). Difference in the subject age also does not appear to be an important factor leading to the different results of these studies as we showed that cytokine mRNA expression and age were not related (Figure 2.4). Examining cytokine levels in a single study group using the varied methods of all these investigations may clarify the reasons for the different conclusions. Moreover, a long-term study from early childhood to adulthood would illuminate whether there is a stage-specific switch in the Th1-Th2 response observed in autism.

Our results extend a growing body of observations that relate abnormal immune functions to autism. Altered helper T cell function has also been reported in other pediatric psychiatric diseases, including childhood-onset schizophrenia, obsessive-compulsive disorder and attention deficit hyperactivity disorder (Mittleman *et al.*, 1997). One possible mechanism linking Th1 CD4+ T-cell activation in the peripheral blood and autism is the ability of helper T cells to cross the blood-brain barrier (Wekerle *et al.*, 1986, 1994). Once within the central nervous system these cells could trigger an

inflammatory response that may damage areas of the brain critical to the development of autism. Although the work reported here does not allow drawing the conclusion that a causal link exists between the Th1 immune response and autism, the repeated association between autism and immune system abnormalities demands closer examination of this central question.

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CHAPTER 3

PREFERENTIAL EXPRESSION OF THE T CELL RECEPTOR V-β CHAIN SUBTYPE V-β 13 IN AUTISM

ABSTRACT

T cell-mediated immune abnormalities are associated with autism. The possibility that specific T cell clones are expanded in autism was investigated in this work by analyzing the expression of the 24 major T cell receptor (TCR) V-β chains in a group of 11 autistic and 9 normal subjects. The expression of these V-β chains serves as a marker for T cell clone expansion. Our results indicated that autistic subjects predominantly expressed V-β 13. The difference in V-β 13 expression relative to the control group was significant ($p < 0.01$; Wilcoxon's rank sum test). The expression of V-β 13 was especially prevalent in the subgroup of autistic individuals who carried the DR4 or DR1 alleles that are common in autism. V-β 19 and 22 are underrepresented ($p < 0.01$ and $p < 0.05$, respectively) in the autistic group relative to the controls. This is the first report of an alteration of the TCR repertoire in autism and is consistent with many previous reports that immune system abnormalities are involved in autism. The prevalence of expanded V-β 13-expressing T cell clones strengthens evidence that at least some cases of autism are a result of an autoimmune disorder.

INTRODUCTION

Multiple causes have been postulated for the etiology of autism, including genetic factors, immune dysfunction (Warren *et al.*, 1986), and viral infections (Chess, 1977;

Deykin and Macmahon, 1979; Markowitz, 1983). Immune abnormalities, especially T cell-mediated dysfunction, have been intensively investigated in autism. Relevant findings associated with autism include increased serum levels of interleukin type 2 (IL-2), interleukin type 12 (IL-12) and interferon-γ (Singh, 1996), decreased T cell responses to mitogens (Stubbs *et al.*, 1977), depressed CD4+ T cell functions (Yonk *et al.*, 1990), decreased natural killer functions (Warren *et al.*, 1990), and increased expression of the DR+ surface marker (Warren *et al.*, 1990; Plioplys *et al.*, 1994).

T cell clone expansion is a hallmark of autoimmune diseases. A panel of these documented autoimmune diseases with T cell clone expansion includes multiple sclerosis (Wucherpfennig and Hafler, 1995; Lozeron *et al.*, 1998), type I diabetes (Atlan-Gepner *et al.*, 1997), systemic lupus erythematosus (Furukawa *et al.*, 1996; Holbrook *et al.*, 1996; Mato *et al.*, 1997), Kawasaki disease (Abe *et al.*, 1993), sarcoidosis (Moller *et al.*, 1988), microscopic polyarteritis (Simpson *et al.*, 1995), and rheumatoid arthritis (Jenkins *et al.*, 1996; Davey and Munkirs, 1993). In every one of these disorders, one or a few T cell clones form the predominant activated T cell population. Many other diseases, including autism, are suspected to have an autoimmune basis and show T cell clone expansion.

Expanded T cell clones usually are identified on the basis of the T cell receptors they express. The common T cell receptor is a heterodimer of α and β chains. The specificity of antigen interaction with the TCR is controlled largely by the variable region (V) of the β chain (Wucherpfennig and Hafler, 1995). Characteristic patterns of T-cell V-β expression have been associated with autoimmune diseases (McMurray *et al.*, 1996). Two methods are available for the detection of specific V-β chains: flow cytometry and

reverse transcription-based polymerase chain reaction (RT-PCR). RT-PCR has the advantage of greater sensitivity (Than *et al.*, 1997) and now is the method of choice.

The studies reported here use RT-PCR of mRNA isolated from peripheral blood to learn if particular T cells clones are expanded in autism. The expression patterns of the 24 major V-β chains were examined in a group of 11 autistic and 9 normal individuals. V-β 13 was the predominant V-β chain expressed by autistic individuals, indicating the expansion of a subset of T cell clones in many cases of autism. The association between V-β 13 expression and autism was even more pronounced when the subgroups of individuals who were positive for the antigen-presenting DR alleles that are associated with autism (DR4 or DR1) were analyzed separately. The expansion of V-β 13-expressing T cell clones in autistic patients provides further evidence that at least some cases of autism are a result of an autoimmune disorder.

MATERIALS AND METHODS

Study Subjects

This study examined 11 autistic and 9 normal subjects whose gender, age and DR types of the study subjects are summarized in Table 3.1. The 11 autistic subjects were all male with a mean age of 11.5 years and the diagnosis of autism was accomplished by psychiatrists according to criteria for autism of the American Psychiatric Association (1994). The male to female ratio of autistic individuals in the general population is about 4:1 (Shaner, 1997). Seven autistic subjects had the human leukocyte antigen (HLA) alleles DR4 or DR1 that increase susceptibility to autism. The normal subjects (6 male, 3

Table 3.1
Sex, Age and DR Types of the Subjects for V-β Analysis

Autistic Subject	Sex	Age	DR types[a]	Normal Subject	Sex	Age	DR types[a]
#1	M	6	3, 4	#1	M	8	9, 12
#2	M	10	1, 4	#2	F	10	3, 4
#3	M	10	4, 15	#3	F	10	1, 7
#4	M	10	4, 7	#4	M	10	3, 13
#5	M	11	1, 11	#5	M	11	4, 12
#6	M	11	3, 10	#6	F	12	1, 15
#7	M	13	4, 13	#7	M	13	11, 12
#8	M	13	3, 10	#8	M	14	4, 15
#9	M	14	12, 13	#9	M	15	7, 9
#10	M	14	7, 16				
#11	M	15	4, 13				

[a]Numbers represent HLA DR types in short format where 1 = DR1, 3 = DR3, 4 = DR4, *etc*.

female) had a mean age of 11.4 years. Five of these normal subjects had either the DR4 or DR1 allele. Child psychiatrists diagnosed autism according to diagnostic parameters of American Psychiatric Association (1994).

HLA Typing

Blood was collected into heparin-coated tubes and peripheral blood mononuclear cells (PBMC) were isolated by histopaque centrifugation. HLA typing was performed by the microlymphocytotoxicity assay using a kit from One Lambda, Inc. (Canoga Park, CA).

Total RNA Isolation and cDNA Synthesis

PBMC isolated by Ficoll-Hypaque centrifugation were stimulated with phytohemagglutinin (PHA) (10 μg/ml) (Gibco, BRL). The cells were cultured in the presence of PHA at 37°C in a humidified 5% CO_2 atmosphere for 5 days before extraction of total RNA. RNA was extracted from 5 x 10^5 PBMC using a kit according to manufacturer's instructions (Trizol reagent kit, Gibco, Gaitherburg, MD). The resulting total RNA was dissolved in 30 μl of sterile H_2O and its concentration was determined by absorbance at OD_{260nm}.

To synthesize cDNA, 2 μg of total RNA was reverse-transcribed using a kit according to manufacturer's instructions (Boehringer, Mannheim, GmbH, Germany). The 20-μl reaction contained 0.1 OD_{260} of random hexamers, 40 units of RNase inhibitor, 1 mM of each dNTP, 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 200 units of Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega,

Madison, WI). The mixture was incubated at 37°C for 2 hours.

Precautions were taken to avoid mRNA degradation and contamination. cDNA synthesis was always performed immediately following RNA isolation to minimize potential for mRNA degradation. An RNase inhibitor (Promega, Madison, WI) was also added at 1 unit/μl to the cDNA synthesis reaction. Solutions, glassware, pipets, and bench surfaces were treated with diethyl pyrocarbonate to inactivate RNases.

TCR V-β Chain Expression

The cDNA resulting from reverse transcription was divided equally into 24 tubes, each containing a 5' TCR V-β-specific sense primer, a common 3' primer that annealed to the constant region of the β chain, and 0.1 pg of competitor cDNAs. The competitor cDNAs were kindly provided by Dr. D. Spinella (Chugai Biopharmacetics, San Diego, CA) and served as internal controls of primer efficiency (Spinella and Robertson, 1994). The 24 competitor cDNAs were constructed by inserting an 86-bp *Xho* II fragment from pUC 19 into a unique *BgI* II site in the constant region of the β chain.

PCR was performed in a 15-μl reaction that contained 10 mM Tris (pH 8.3), 50 mM KCl, 1.2 mM $MgCl_2$, 1 mM of each dNTP, 1 unit Taq DNA polymerase (Fisher Scientific, Pittsburgh, PA), and 45 pmol of each primer. PCR was carried out with an initial 5 minutes of denaturation at 94 °C, followed by 30 cycles of the profile: 1 minute at 94 °C, 1 minute at 58 °C, and 1 minute at 72 °C. After the PCR, 4 μl of the reaction was analyzed by electrophoresis through a 2% agarose gel. The gel was stained with ethidium bromide and photographed under UV light, and the intensity of each band in the photograph was quantified using Biosoft Image System (Biosoft, Ferguson, MO).

The relative expression of each V-β chain was determined according to Spinella and Robertson (1994). A value of 1.0 was assigned to the most intense competitor band obtained in the 24 PCR reactions run from a single individual and all other 23 competitor bands were normalized relative to this intensity value. These ratios represent the amplification efficiency of the 24 primer pairs. Next, the intensity of the endogenous V-β chain cDNA product was divided by the amplification efficiency of the primer pair. These 24 values are referred to as corrected expression. Corrected expression values were normalized relative to the highest corrected expression value in the set of 24 V-β chains. Each normalized value is referred to as a relative expression index (REI) and provides an estimate of the relative amount of each V-β chain mRNA expressed in an individual. Negative controls lacking template DNA were included in all PCR analyses. In no case was there evidence of spurious amplification.

Wilcoxon's rank sum test (Harnett, 1982) was applied to estimate the significance of differences in the rank order of V-β chain expression between the autistic and normal groups.

Cytokine Expression

Interferon-γ and interleukin type 4 (IL-4) mRNA levels were determined using kits supplied by Clontech (Palo Alto, CA). Two μl of cDNA synthesized from PBMC mRNA was placed into five tubes. Each tube contained a cytokine-specific primer pair and 2 μl of a member of a 1:3 dilution series of competitor cDNA. Five independent reactions were performed using conditions described above for each subject for each cytokine (see Chapter 2). The IL-2 mRNA levels were measured relative to actin mRNA

according the method of Haraguchi *et al.* (1995). Actin mRNA expression was assumed to be constitutive. Separate PCRs were performed to amplify IL-2 and actin. The PCR products were analyzed by electrophoresis through a 1.5% agarose gel and photographed under UV light. The amount of IL-4 and interferon-γ mRNA was determined by finding a lane in which the intensities of the competitor and endogenous cDNA products matched. This occurred when the amounts of starting competitor and endogenous cDNA were equal. Since there was a known amount of competitor cDNA added to each reaction, this gave the amount of starting endogenous cDNA. Relative expression levels of IL-2 were determined by comparison between the intensity of the amplification products of the IL-2 and actin mRNAs from the same individual. This ratio was termed arbitrary optical density units (AODU). Student's t test was used to analyze the significance of differences between the average AODU in the autistic and normal groups.

RESULTS

Competitive RT-PCR was used to study TCR V-β expression in autistic subjects. Figure 3.1 shows a representative gel displaying RT-PCR products of TCR V-β chain mRNA from a autistic subject. Two DNA bands are visible in each lane of the gel (Figure 3.1). The smaller band is generated from target cDNA and the larger one is derived from competitor cDNA. As expected, the products from competitor cDNAs were 86-bp larger than the corresponding products of endogenous mRNAs. The REI values for each V-β derived from analysis of this gel are shown in Figure 3.2. V-β 3 and V-β 13 are the predominant V-β forms expressed in this individual. This indicates that there was an

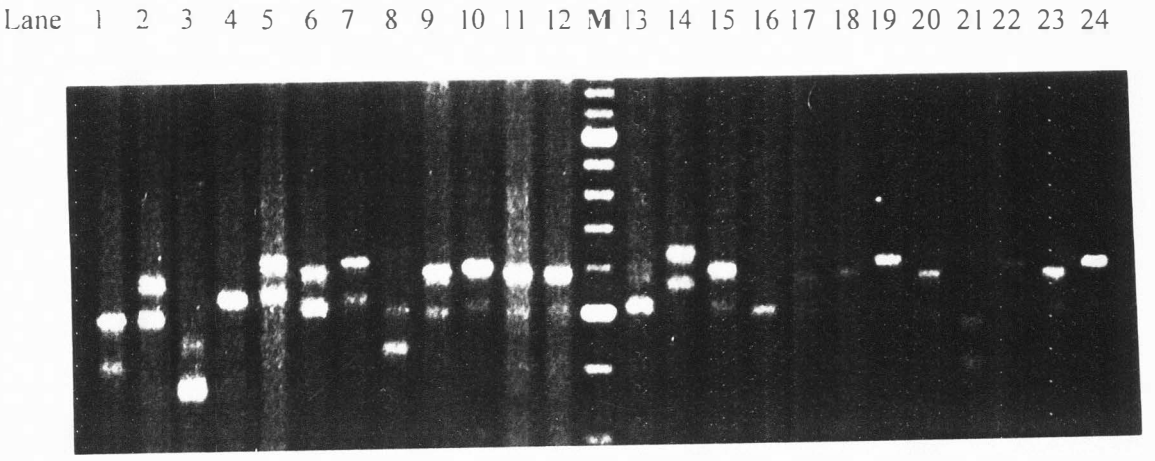

Fig. 3.1. RT-PCR analysis of V-β chain expression in a single individual.

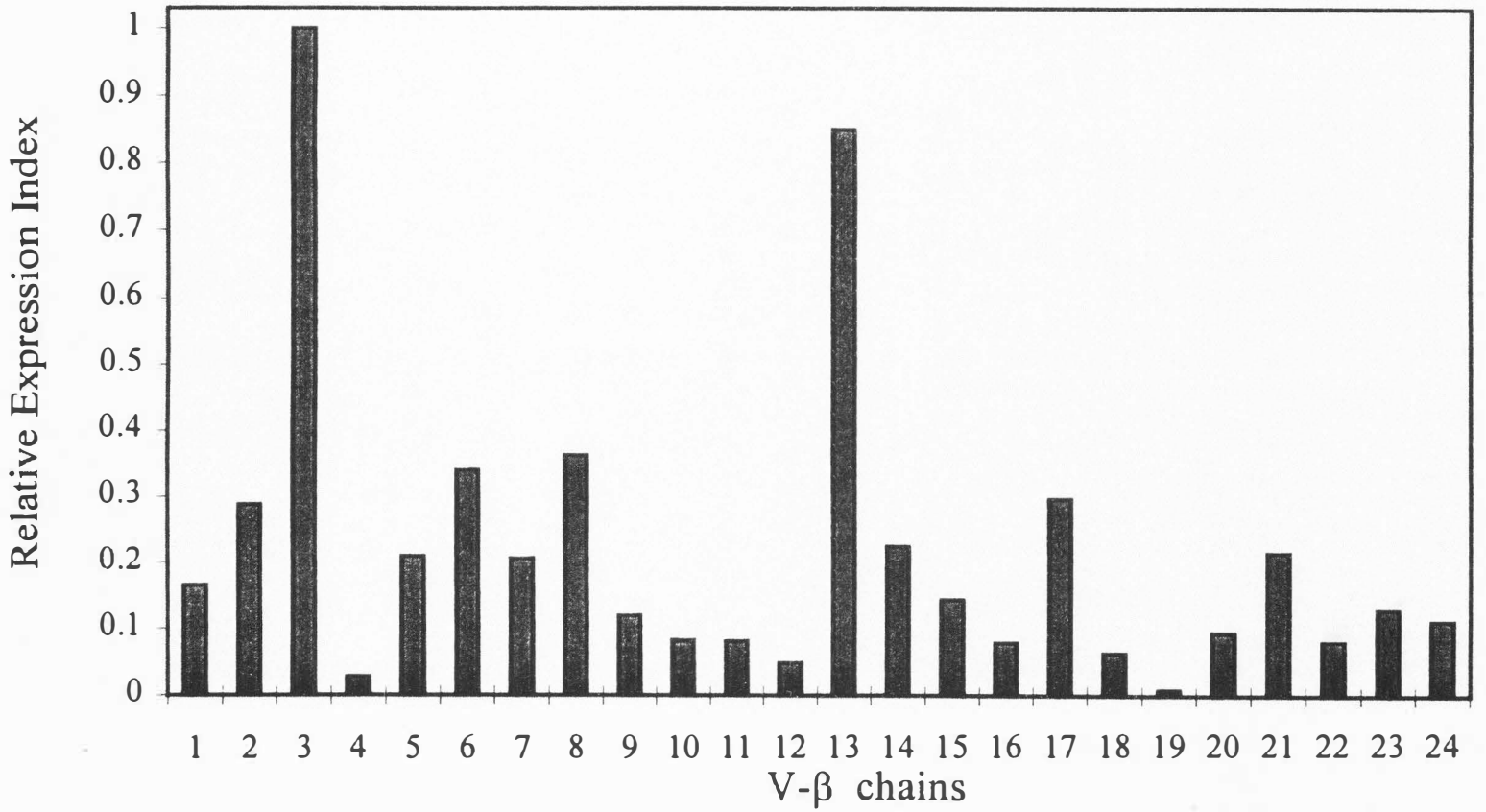

Fig. 3.2. Relative V-β expression in the same individual examined in figure 3.1.

expansion of T cells clones that express these forms of the TCR β chain in this individual. Similar analyses were performed for all subjects of the study.

The mean REI for each V-β chain was calculated in the autistic and normal groups. These values were compared between the groups and are shown in Table 3.2. Inspection of the results shows that in the autistic group V-β 13 was found at appreciably higher frequency and V-β 14, 15, 19, and 22 were found at appreciably lower frequencies. The significance of these differences was analyzed by Wilcoxon's rank sum test, a statistical test that compares the rank order of V-β chain expression between the two groups, according to the methods of Jenkins *et al.* (1996). As shown in Table 3.2, there was a significant increase of V-β 13 expression ($p < 0.01$) and a significant decrease in V-β 19 ($p < 0.01$) and V-β 22 ($p < 0.05$) in the autistic subjects. Differences in the relative expression of other V-β chains between groups were not significant according to this measure.

Individuals who possess either the DR4 or DR1 allele are at greater risk of developing autism (Warren *et al.*, 1992). This led us to examine whether V-β chain expression patterns would differ between the subgroups of autistic and normal subjects who express either DR4 or DR1. Application of Wilcoxon's rank test showed that there was significantly increased expression of V-β 13 in autistic subjects ($n = 7$; $p < 0.01$) relative to normal subjects ($n = 5$) with the mean REI of 0.86 for V-β 13 in the autistic group and 0.15 in the normal group. In this comparison of subgroups of DR4 or DR1 positive individuals, there was no significant difference in the relative expression of V-β 19 and 22 as seen in the comparison of all autistic and normal individuals (Table 3.3). These results showing significant expansion of V-β expressing T cells clones in autistic

Table 3.2
TCR V-β Expression in Autistic and Normal Subjects

V-β	Autistic (n = 11) REI[a] (μ ± S.D.[b])	Normal (n = 9) REI[a](μ ± S.D.[b])	Wilcoxon's Rank Sum Test
1	0.18 ± 0.18	0.19 ± 0.18	
2	0.27 ± 0.18	0.30 ± 0.34	
3	0.33 ± 0.33	0.33 ± 0.41	
4	0.06 ± 0.06	0.14 ± 0.32	
5	0.31 ± 0.28	0.33 ± 0.39	
6	0.47 ± 0.31	0.32 ± 0.24	
7	0.18 ± 0.13	0.19 ± 0.16	
8	0.31 ± 0.29	0.32 ± 0.32	
9	0.23 ± 0.28	0.21 ± 0.22	
10	0.08 ± 0.08	0.08 ± 0.12	
11	0.11 ± 0.15	0.07 ± 0.09	
12	0.10 ± 0.11	0.08 ± 0.07	
13	**0.92 ± 0.17**	**0.34 ± 0.39**	**$p < 0.01$[c]**
14	0.12 ± 0.12	0.29 ± 0.28	
15	0.07 ± 0.09	0.24 ± 0.32	
16	0.17 ± 0.19	0.06 ± 0.06	
17	0.12 ± 0.12	0.18 ± 0.31	
18	0.08 ± 0.09	0.06 ± 0.06	
19	**0.02 ± 0.03**	**0.06 ± 0.06**	**$p < 0.01$[c]**
20	0.08 ± 0.14	0.15 ± 0.16	
21	0.33 ± 0.28	0.29 ± 0.26	
22	**0.06 ± 0.08**	**0.25 ± 0.22**	**$p < 0.05$[c]**
23	0.22 ± 0.23	0.21 ± 0.23	
24	0.05 ± 0.05	0.08 ± 0.14	

[a]Relative expression index.

[b]Mean ± standard deviation.

[c]Only values that indicate a significant difference between group are shown.

Table 3.3
TCR V-β Expression in Autistic and Normal Subjects Expressing DR4 or DR1

V-β	Autistic (n = 7) REI[a] (μ ± S.D.[b])	Normal (n = 5) REI[a] (μ ± S.D.[b])
1	0.19 ± 0.22	0.16 ± 0.18
2	0.24 ± 0.22	0.36 ± 0.40
3	0.31 ± 0.39	0.34 ± 0.41
4	0.07 ± 0.08	0.22 ± 0.44
5	0.26 ± 0.31	0.26 ± 0.42
6	0.50 ± 0.36	0.28 ± 0.25
7	0.17 ± 0.16	0.15 ± 0.13
8	0.34 ± 0.37	0.35 ± 0.35
9	0.26 ± 0.35	0.15 ± 0.16
10	0.09 ± 0.09	0.06 ± 0.08
11	0.15 ± 0.18	0.05 ± 0.06
12	0.12 ± 0.14	0.07 ± 0.05
13	**0.86 ± 0.20[c]**	**0.15 ± 0.15[c]**
14	0.11 ± 0.13	0.23 ± 0.21
15	0.09 ± 0.10	0.26 ± 0.42
16	0.19 ± 0.23	0.03 ± 0.03
17	0.13 ± 0.12	0.04 ± 0.02
18	0.09 ± 0.10	0.06 ± 0.06
19	0.03 ± 0.04	0.05 ± 0.04
20	0.11 ± 0.17	0.12 ± 0.16
21	0.37 ± 0.34	0.15 ± 0.11
22	0.09 ± 0.09	0.26 ± 0.22
23	0.30 ± 0.25	0.14 ± 0.10
24	0.06 ± 0.06	0.03 ± 0.04

[a]Relative expression index.
[b]Mean ± standard deviation.
[c]Highly significant difference ($p < 0.01$; Wilcoxon's rank sum test).

individuals correlate well with a previous finding that showed activation of Th1 cells in the disease (see Chapter 2).

Six of the autistic subjects in this study were coparticipants in the study that examined the profiles of IL-2, interferon-γ and IL-4 expression as markers for activation of Th1 or Th2 cells (Chapter 2). A cross-study comparison was made using this group of autistic subjects to learn if the correlation between the expansion of V-β 13-expresssing T cell clones and elevated expression of Th1-specific cytokines (interferon-γ and IL-2) occurred in the same individuals. The relative expression level of V-β 13 was significantly higher in this group of autistic individuals (n = 6) (see Table 3.4) but not in the normal controls (n = 9). As shown in Table 3.4, levels of the Th1-specific cytokines IL-2 and interferon-γ are also elevated in the same group of autistic individuals. This correlation of expansion of V-β 13-expresssing T cell clones and elevated expression of IL-2 and interferon-γ in the same set of autistic individuals suggests that both these processes may cooperatively interact in the pathogenesis of autism.

DISCUSSION

In this study, we showed that in many autistic individuals elevated V-β 13 chain expression is elevated. This is the first report of an alteration of the TCR repertoire associated with autism. This finding is consistent with many previous reports of altered immune responses in autism. Expression of particular T cell clones occurs in many autoimmune diseases (Marchalonis *et al.*, 1994; Schneider and Gronvik, 1995; Wooley and Cingel, 1995), and our finding of V-β 13 expansion in autism provides evidence that at least some cases of autism are a result of autoimmunity.

Table 3.4

Cytokine and V-β Chain Expression in Six Autistic Subjects

Group	Subject	Sex	DR Types	Age (years)	IL-2/Actin[a]	Interferon-γ mRNA Molecules per μg of Total RNA	V-β 13 (REI[b])
Autistic	#1	M	4, 7	19	0.85	19,293	1.00
	#2	M	1, 4	9	0.42	N.D.[c]	1.00
	#3	M	12, 13	15	0.31	N.D.[c]	0.50
	#4	M	1, 11	11	0.65	19,293	1.00
	#5	M	3, 10	11	1.33	519,280	1.00
	#6	M	4, 13	13	0.05	19,293	0.80
Autistic subjects (μ ± SD[d])					0.58 ± 0.45 (n = 6)	15,900 ± 15,900 (n = 6)	0.88 ± 0.20 (n = 6)
Normal subjects (μ ± SD[d])					0.18 ± 0.20 (n = 19)	4,500 ± 8,200 (n = 19)	0.34 ± 0.39 (n = 11)
Student's t test					$p < 0.01$	$p < 0.01$	$p < 0.01$

[a]IL-2 mRNA levels relative to actin mRNA levels.

[b]Relative expression index.

[c]Not detectable in the assay.

[d]Mean ± standard deviation.

The TCR repertoire is determined by many factors, including genetic factors, major histocompatibility complex (MHC)-mediated selection by antigens during T cell development in the thymus, and by infection (Islam *et al.*, 1996). TCR repertoires are significantly altered in many autoimmune disorders (Marchalonis *et al.*, 1994; Schneider and Gronvik, 1995; Wooley and Cingel, 1995). For example, in rheumatoid arthritis, V-β 17 expression predominates (Davey and Munkirs, 1993; Fischer *et al.*, 1996), and in multiple sclerosis T cell clones that express V-β 8 predominant (Wucherpfennig and Hafler, 1995). It is tempting to speculate that a similar link exists between the expansion of V-β 13-expressing T cell clones and the development of autism.

The expression of V-β 13 was especially prevalent in the group of autistic individuals who carried the DR4 or DR1 alleles that are common in autism. In many autoimmune diseases, including insulin-dependent diabetes mellitus, rheumatoid arthritis, and multiple sclerosis, there is a strong association between the disease and the expression of a particular DR molecule (Geha *et al.*, 1994). These autoimmune diseases also show preferential expression of particular V-β chains that indicate expansion of specific T cell clones (Grunewald *et al.*, 1998; Navaneetham *et al.*, 1998; McKee *et al.*, 1999). One model that links these observations postulates that specific DR molecules present some antigens in a way that is recognized preferentially by a single form of TCR. If the DR-antigen complex mimics a self-antigen, the T cell clone that is expanded by stimulation with a foreign antigen could produce an autoimmune response that targets host tissue specific to the disease. This may be the case in the relationship between DR4 or DR1 and V-β 13-expressing T cells in autism.

There is a correlation between dominant expression of V-β 13 and the expression of Th1-specific cytokines IL-2 and interferon-γ in the 6 autistic individuals who participated in this study and an earlier investigation of cytokine expression study (Chapter 2). Enhanced cytokine expression and expansion of T cells that express V-β 13 may be tied together in a positive feedback loop in which the cytokines stimulate T cell proliferation and in turn the T cells produce yet more Th1 specific cytokines. If V-β 13-expressing T cells are involved in an autoimmune response in autism, elevated cytokine expression is likely to exacerbate symptoms of the disease.

The identification of V-β 13 as the predominant V-β chain expressed in most autistic individuals may aid in the identification of autoantigens that are targeted in the disease. One approach to this goal would be to isolate the V-β 13-expressing T cell clones from autistic patients and test their ability to be stimulated by self-antigens.

If the finding of an association between autism and expansion of T cell clones expressing V-β 13 is general, then there is the possibility for immune therapy based on deletion of these specific T cell clones. Such an approach has been successfully demonstrated in animal models of multiple sclerosis, where treatment with antibody directed against the predominant TCR V-β 8 chain dramatically reduced mortality associated with the disease (Gold *et al.*, 1997; Vandenbark *et al.*, 1992).

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CHAPTER 4

INVESTIGATION OF T CELL RESPONSE TO EPITOPES OF SUSPECTED PATHOGENS IN AUTISTIC PATIENTS

ABSTRACT

This communication reports tests of two models for autism that are based on T cell-mediated immune abnormalities. One model postulates that infections in compromised individuals allow pathogen persistence to cause central nervous system (CNS) damage that leads to autism. A prediction of this model is that autistic individuals will show a reduced immune response to the pathogen that triggered the disease. T cell epitopes derived from pathogens suspected to cause autism were examined for their ability to induce T cell proliferation in autistic and normal subjects. As a group, the T cells of autistic subjects did not show an altered response to peptides derived from rubella virus, herpes simplex virus type 1 (HSV-1), cytomegalovirus (CMV), Epstein-Barr virus (EBV), and *Clostridium tetani*. Increased T cell proliferation in autistic individuals to a peptide derived from influenza A virus was noted. These results do not support a model in which autism is caused by an immune tolerant response to any of the pathogens tested here. Another model of autism tested here is that autism is induced by pathogens that possess epitopes identical to the hypervariable region 3 (HVR-3) of the human leukocyte antigen (HLA) DR4 or DR1 alleles that are prevalent in autism. This model proposes that T cell-mediated immunity is ineffective against these pathogens so they persist and ultimately cause CNS damage that produce autism. Peptides derived from the *Escherichia coli (E. coli) dna* J and the Epstein-Barr virus (EBV) glycoprotein (gp) 110

that contain sequences identical to the HVR-3 of the DR4 and DR1 alleles were examined for their ability to induce T cell proliferation in DR4 or DR1 positive and negative autistic and normal subjects. There was no relationship between possession of DR4 or DR1 allele and the ability to respond to either peptide. Although the results of these studies do not provide support for involvement of a specific pathogen in autism, the average T cell proliferative activity of autistic subjects was lower than that of normal individuals. This result is consistent with prior reports that show there is a reduced T cell-mediated immune response in autism.

INTRODUCTION

Infections have long been associated with autism (Chess, 1977; Stubbs, 1976), although the mechanism that couples infections with the development of autism is unknown. Pathogens associated with autism by epidemiological investigations mainly include rubella virus, HSV-1, and CMV. In addition, immunization with rubella and tetanus vaccines has been proposed to cause autism in a small number of individuals (Munyer *et al.*, 1975; Rossier *et al.*, 1977). A potential link between infection and autism is the depression of immune functions that is often seen in autistic subjects. These reduced immune system functions include lower numbers of CD4+ T cells (Yonk *et al.*, 1990; Warren *et al.*, 1990), decreased amounts of complement C4B (Warren *et al.*, 1995), reduced responsiveness to mitogens (Yonk *et al.*, 1990; Warren *et al.*, 1986), depressed functions of natural killer cells (Warren *et al.*, 1987), and abnormal cytokine profiles (Singh *et al.*, 1991; Singh, 1996; Gupta *et al.*, 1998) indicative of defective T cell functions. One model that relates infection and autism postulates that if pre- or postnatal

infections occur in individuals with a reduced ability to clear the infectious agent, this will allow the pathogen to persist and cause CNS damage that leads to autism. One prediction of this model is that autistic individuals will show a reduced T cell-mediated immune response to the pathogen that triggered the disease. This prediction was tested here by assessing the T cell proliferation responses of autistic and normal subjects to a set of peptide epitopes derived from pathogens suspected to cause the disease.

HLA DR molecule-mediated autoimmunity may be involved in some cases of autism. For this reason, another model of autism is based on understanding of the autoimmune disorder rheumatoid arthritis (RA) and the roles played by specific HLA DR alleles. HLA DR molecules are T cell surface proteins involved in antigen presentation (Geha *et al.,* 1994; Auger *et al.*, 1996). In both RA and autism, individuals who possess either the HLA DR4 or DR1 allele are susceptible to the diseases (Albani *et al.*, 1992a; Warren *et al.*, 1992; Daniels *et al.*, 1995). DR proteins contain a set of allele-specific sequences known as hypervariable regions that have key roles in antigen binding and presentation, with the third hypervariable region (HVR-3) exerting a predominant effect (Geha *et al.,* 1994). In RA, it is postulated that pathogens that display antigens with regions similar to the HVR-3 induce immune tolerance (Salvat *et al.*, 1994). It is well noted that the *E. coli dna* J protein and EBV gp110 possessed a sequence identical to the core pentapeptide sequence (QKRAA) of the HVR-3 of HLA DR4 and DR1 (Albani *et al.*, 1992b). These *E. coli dna* J and EBV gp110 proteins appear to be important antigenic determinants associated with RA as a high titer of antibodies directed against them is found in the synovial fluid of RA patients (Takeuchi *et al.*, 1990). Moreover, *dna* J and gp110 act as potent inducers of T cell proliferation in both *in vitro* and *in vivo* assays

(Ikeda *et al.*, 1996; Roudier *et al.*, 1988, 1989). When DR4- or DR1-positive RA patients were tested for their T cell proliferative responses induced by peptides derived from the *E. coli dna* J protein and EBV gp110 that contained the QKRAA sequence, there was a significantly reduced response relative to RA patients and normal controls who expressed other DR alleles (Salvat *et al.*, 1994). Based on these results, Salvat and his coworkers proposed that some cases of RA may be caused by infections with EBV or *E. coli* that are ineffectively cleared in HLA DR4- or DR1-positive individuals (Salvat *et al.*, 1994). Given the parallel between possession of HLA DR4 and DR1 and susceptibility to RA and autism, we explored the possibility that DR4- or DR1-positive autistic individuals would show a reduced T cell proliferative response to *dna* J and gp110 peptides that contain the QKRAA sequence. This communication reports the results of tests of both models of autism.

MATERIALS AND METHODS

Synthesis of T-Cell-Epitope-Derived Peptides

Ten peptides that represent the major T cell epitopes of pathogens suspected in the etiology of autism were synthesized and purified by high performance liquid chromatograph at the Utah State University Biotechnology Center. Table 4.1 lists the amino acid sequences of these peptides.

Subjects Studied

The sex, age, and HLA DR types of the 24 autistic and 18 normal subjects

Table 4.1

Peptides Used in the T Cell Proliferation Assay

Peptide[a]	Sequence
Rubella capsid (9-29)	MEDLQKALEAQSRALRAELAA
Rubella E1 (262-283)	DPLLRTAPGPGEVWTPVIGSQ
Rubella E2 (51-75)	YRNASDVLPGHWLQEEWGCYN
HSV gC-1 (128-140)	NRRDPLARYGSRC
HSV gD-1 (5-23)	ADASLKMADPNRFRGKDLP
CMV gB (618-628)	FIAGNSAYEYV
Influenza M (58-66)	GILGFVFTL
Tetanus toxin (947-968)	FNNFTVSFWLRVPKVSASHLEG
EBV gp110 (803-818)	EQNQEQKRAA[b]QRAAGC
E. coli dna J (56-75)	VLTDSQKRAA[b]YDQYGHAAF

[a]Numbers in parantheses indicate the amino acid position in the proteins

[b]The underlined sequences indicate the amino acid sequence shared with the HVR-3 region of HLA types DR4 and DR1.

included in this study are shown in Table 4.2. The diagnosis of autism was accomplished by psychiatrists according to criteria for autism of the American Psychiatric Association (1994). At the time of blood collection, there was no record that any autistic or normal subject received medications, had concurrent medical conditions that might affect the results of the study, or showed signs of infection or allergy. The studies were approved by the Utah State University Human Subject Review Board and written consent from study subjects or their parents was obtained.

T Cell Proliferation Assay

Peripheral blood mononuclear cells (PBMC) from fresh venous blood of each subject were isolated by histopaque density centrifugation. In outline, the T cell proliferation assay was based on previously published methods (Chain *et al.*, 1987; Orlik and Spiltter, 1996) that were optimized in this laboratory (see Appendix B). These final conditions were used for all peptides in this study. For the assay, 5×10^6 PBMC freshly isolated from the same subject were placed in triplicate wells of 96-well flat-bottomed microculture plates (Cell Walls™, Corning, New York) and incubated at 37°C in a humidified 5% CO_2 atmosphere. Peptides at a final concentration of 1 μM were added (see Appendix B). Triplicate negative controls without peptide addition were included in all experiments. One day later, recombinant interleukin type 2 (IL-2) was added to a final concentration of 30 international units/ml (Chiron, Emeryville, CA) (see Appendix B). Five days after starting the assay, 2 μl of ^{3}H-thymidine (1 μCi; Moravek Biochemicals. Inc., Brea, CA) was added to each well. The cells were incubated 3 hours, then transferred by vacuum filtration to a membrane using a cell transfer device (Micromate-

Table 4.2

Sex, Age and HLA DR Types of the Study Subjects

Autistic	Sex	Age	HLA DR	Normal	Sex	Age	HLA DR
#1	M	3	7, 7	#1	M	3	7, 7
#2	M	4	1, 13	#2	F	4	4, 7
#3	M	5	7, 15	#3	M	5	11, 13
#4	F	6	3, 3	#4	M	8	7, 7
#5	M	6	1, 13	#5	M	8	1, 3
#6	M	6	3, 7	#6	M	8	7, 11
#7	M	9	4, 16	#7	F	9	13, 15
#8	F	10	7, 13	#8	M	10	3, 10
#9	M	10	7, 13	#9	M	14	11, 14
#10	M	11	1, 15	#10	M	15	11, 13
#11	M	11	7, 7	#11	M	15	4, 8
#12	M	12	1, 8	#12	F	16	4, 7
#13	F	13	4, 4	#13	M	16	13, 15
#14	F	14	3, 4	#14	M	16	11, 13
#15	M	16	4, 15	#15	M	18	3, 13
#16	F	16	1, 4	#16	M	18	1, 13
#17	M	17	1, 7	#17	M	29	13, 15
#18	M	19	4, 15	#18	M	41	1, 3
#19	M	20	4, 7				
#20	M	23	4, 4				
#21	M	25	1, 7				
#22	M	26	4, 15				
#23	M	27	1, 7				
#24	M	39	4, 15				

196, Packard Instrument Inc., Dower Grove, IL). Unincorporated ^{3}H-thymidine was removed by washing cells on the membrane and the incorporated counts were determined using a β-counter (Matrix^{TM}-96, Packard Instrument Inc., Dower Grove, IL). The average counts per minute (cpm) for triplicate wells analyzed for each condition (10 peptides and no peptide control) was calculated. A stimulation index (SI) for each peptide was calculated as the ratio of the average cpm incorporated in the presence of peptide over the average cpm of the no-peptide control in the same individual.

Statistical Analysis

The SI values were grouped into categories of positive response (SI > 1.95), no response ($1.95 \geq SI \geq 0.5$), and negative response (SI < 0.5). The cutoffs of SI > 1.95 and SI < 0.5 are according to Schrier *et al.* (1995). χ^2analysis was performed to examine the significance of differences between the autistic and normal groups. The results were also analyzed on the basis of absolute cpm incorporated in the T cell proliferation assay. In this case, Student's t test was used.

RESULTS

Response to T-Cell Epitopes of Pathogens Implicated in Autism

PBMC obtained from a group of 24 autistic and 18 normal controls were challenged with 8 synthetic T cell epitopes of 5 pathogens (rubella virus, HSV-1, CMV, influenza A virus, and *Clostridium tetani*) that were implicated as potential causative factors in autism (Deykin and Macmahon, 1979). The proteins used to derive the synthetic T cell epitopes were the rubella virus caspid (C), envelop (E1), and envelop

(E2) (McCarthy *et al.*, 1993), HSV-1 glycoprotein C-1 (gC-1) and glycoprotein D-1 (gD-1) (Jayaraman *et al.*, 1993), CMV glycoprotein B (gB) (Mayer *et al.*, 1996), the influenza A virus matrix (M) protein (Mayer *et al.*, 1996), and the *Clostridium tetani* toxin (Valmori *et al.*, 1994), whose antigenecities are well demonstrated.

The T cell proliferation induced by each peptide in each subject was expressed as SI, the ratio of ^{3}H-thymidine incorporated by PBMC exposed to peptide relative to the ^{3}H-thymidine incorporated by control cells not exposed to peptide. SI values ranged widely for each peptide in both autistic and normal subjects. To facilitate comparisons between the autistic and normal groups, the method of Schrier *et al.* (1995) was followed in which SI values are classified into one of three categories: a positive response to the peptide ($SI > 1.95$), no response to the peptide ($1.95 \geq SI \geq 0.5$), and a negative response to the peptide ($SI < 0.5$). This simplification of results allowed testing of ranked T cell proliferative response by χ^2 analysis.

The analysis of results for all eight peptides in autistic and normal subjects indicated that there was no reduction in T cell proliferation induced by any peptide in the autistic group (Table 4.3). The only difference between the autistic and normal groups was an increased response to the influenza A virus M protein in the autistic group (Table 4.3)

The results of peptide challenge to PBMC of autistic and normal subjects were also analyzed by comparing the mean cpm of the ^{3}H-thymidine incorporated both in the presence or absence of added peptides in the autistic and normal groups. As shown in Table 4.4, for all peptides the PBMC of autistic subjects incorporate less ^{3}H-thymidine than the equivalent number of PBMC of normal subjects under identical assay conditions.

Table 4.3

Ranked T Cell Proliferation Response to Eight T Cell Epitopes

Peptide	Autistic Subjects (n = 24)			Normal Subjects (n = 18)			χ^2	p value
	$SI^a > 1.98$	$1.95 \geq SI^a \geq 0.5$	$SI^a < 0.5$	$SI^a > 1.98$	$1.95 \geq SI^a \geq 0.5$	$SI^a < 0.5$		
Rubella capsid (9-29)	6	13	5	4	12	2	0.89	$p > 0.05$
Rubella E1 (262-283)	4	16	4	3	13	2	0.27	$p > 0.05$
Rubella E2 (51-75)	3	17	4	3	12	3	0.15	$p > 0.05$
HSV gC-1 (128-140)	7	14	3	3	13	2	1.00	$p > 0.05$
HSV gD-1 (5-23)	3	18	3	0	16	2	2.51	$p > 0.05$
CMV gB (618-628)	6	15	3	3	13	2	0.50	$p > 0.05$
Influenza A virus M (58-66)	5	13	6	0	16	2	6.59	$p < 0.05$
Tetanus toxin (947-968)	6	14	4	3	11	4	0.51	$p > 0.05$

[a]Stimulation index.

The difference between groups was significant according to Student's t test for all rubella- and HSV-derived peptides. The average ^{3}H-thymidine incorporated by PBMC not exposed to peptide in the autistic and normal groups was actually higher than that observed following treatment with most peptides. This does not indicate that no individuals showed a positive response to peptide exposure, only that on average neither the autistic nor the normal group showed stimulation by peptides. This is consistent with the analysis of this data set by SI values, which shows that roughly equal numbers of individuals of each group respond positively or negatively to any peptide (Table 4.4).

Stimulation with the mitogen, phytohemagglutinin (PHA), was used as a positive control to test the responsiveness of T cells in the PBMC preparations. For both the control and autistic groups, PHA induced a strong response over background levels observed in PBMC without any addition of mitogen (Table 4.4). As previously reported (Stubbs *et al.*, 1977; Warren *et al.*, 1986), the level of ^{3}H-thymidine incorporation in PHA-treated cells was lower in the autistic group relative to normal controls (Table 4.4). However, in contrast to these earlier reports, this difference was not statistically significant.

Responses to Peptides with Sequences Similar to HLA DR4 or DR1

T cell proliferation was examined in response to peptides of the *E. coli dna* J and EBV glycoprotein (gp)110 protiens which contain the pentapeptide sequence QKRAA that is present in the HVR-3 of HLA DR4 or DR1. The average SI value of the DR4- or DR1-positive autistic subjects was compared with the SI value of three different groups: DR4- or DR1-negative autistic individuals (n = 7), DR4- or DR1-postitive normal

Table 4.4

^{3}H-Thymidine Incorporation after Exposure to 10 Peptides in Normal and Autistic Subjects

Peptide	Autistic (n = 24) $\mu \pm SD^{a}$	Normal (n = 18) $\mu \pm SD^{a}$	Student's t Test p value[b]
Rubella capsid (9-29)	303 ± 334	1109 ± 1553	0.041
Rubella E1 (262-283)	253 ± 265	801 ± 832	0.011
Rubella E2 (51-75)	258 ± 264	806 ± 1111	0.049
HSV gC-1 (128-140)	297 ± 278	957 ± 1272	0.040
HSV gD-1 (5-23)	237 ± 268	644 ± 725	0.030
CMV gB (618-628)	479 ± 965	938 ± 1154	0.149
Influenza A virus M (58-66)	261 ± 301	1009 ± 1753	0.086
Tetanus toxin (947-968)	354 ± 424	901 ± 1408	0.107
PHA	3238 ± 3669	4997 ± 5971	0.228
Background	405 ± 706	1054 ± 1283	0.054

[a]Mean ± standard deviation.

[b]The difference between the autistic and normal subjects was determined by Student's t test.

individuals (n = 6), and DR4- or DR1-negative normal individuals (n = 12). There was no significant difference in the average SI values in any comparison (Table 4.5).

The ages of autistic subjects used in this study varied widely (3-39 years). Since age is known to be a factor in the immune response, we investigated whether age played a role in T cell responsiveness to peptides in the current study. Autistic subjects were grouped into categories of prepubescent children and adults using a cutoff age of 14 years. The SI values observed in response to peptide exposure in these age groups are shown (Table 4.6). There was no significant effect of age in response to peptide.

DISCUSSION

Two questions were examined in this study. The first was whether autistic individuals have a compromised T cell-mediated immune response to pathogens reported to be associated with the disease. The second was whether pathogens with T cell epitopes similar to the T cell-interacting HVR-3 of HLA DR4 or DR1 were tolerated in autistic individuals. Both questions were tested by examining the T cell proliferation responses of autistic and normal subjects to a set of peptide epitopes derived from pathogens implicated in autism.

Averaged as a group, T cells of autistic subjects did not show a reduced proliferative response to peptides derived from rubella virus, HSV-1, CMV, influenza A virus, and *Clostridium tetani*. The only difference was an enhanced response to the influenza A virus M protein in autistic subjects. Therefore, these results do not provide support for the idea that a lower immune response to infections or immunization against common viruses plays a significant role in the disease. However, in coming to this

Table 4.5

Proliferation Response to EBV gp110 and *E. coli. dna* J Peptides[a]

Peptide	Autistic Subjects with DR4 or DR1 (n = 17) SI[b] ($\mu \pm SD^c$)	Autistic Subjects without DR4 or DR1 (n = 7) SI[b] ($\mu \pm SD^c$)	Normal Subjects with DR4 or DR1 (n = 6) SI[b] ($\mu \pm SD^c$)	Normal Subjects without DR4 or DR1 (n = 12) SI[b] ($\mu \pm SD^c$)
EBV gp110 (803-818)	1.10 ± 0.73	1.23 ± 1.16	1.75 ± 2.02	1.19 ± 0.65
E. coli dna J (56-75)	1.79 ± 1.06	1.35 ± 1.55	2.90 ± 3.46	1.22 ± 0.66

[a]Student's t test indicated no significant difference in SI between the DR4 or DR1 positive autistic individuals compared to all other groups.

[b]Stimulation index.

[c]Mean ± standard deviation.

Table 4.6
Proliferation Response to 10 Peptides in
Two Age Groups of Autistic Subjects[a]

Peptide	Age < 14 (n = 13) SI[b] ($\mu \pm SD^c$)	Age ≥ 14 (n = 11) SI[b] ($\mu \pm SD^c$)
Rubella capsid (9-29)	1.30 ± 1.05	1.82 ± 1.28
Rubella E1 (262-283)	1.07 ± 0.74	1.44 ± 0.70
Rubella E2 (51-75)	1.12 ± 0.68	1.51 ± 0.76
HSV gC-1 (128-140)	1.58 ± 1.38	1.44 ± 0.65
HSV gD-1 (5-23)	1.11 ± 0.95	1.27 ± 0.85
CMV gB (618-628)	1.92 ± 1.47	1.13 ± 0.40
Influenza A virus M (58-66)	1.21 ± 0.93	1.16 ± 0.73
Tetanus toxin (947-968)	1.33 ± 0.99	1.79 ± 1.42
EBV gp110 (803-818)	1.11 ± 0.91	0.96 ± 0.54
E. coli dna J (56-75)	1.39 ± 1.11	1.71 ± 0.95

[a]Student's t test indicated that there were no significant differences between age groups in response to any peptide.

[b]Stimulation index.

[c]Mean ± standard deviation.

conclusion, it is important to realize that widely varying individual responses were seen in each group. It remains possible, as suggested by epidemiological studies, that some cases of autism are caused by infectious agents. If these cases occur at low frequency, the method of comparison between groups that was used here would fail to detect them.

The second hypothesis examined in this work was the possibility that autism may be induced by pathogens that possess protein antigens that share sequences with the HLA DR4 or DR1. These particular DR types are expressed at much higher frequencies in autistic subjects than in the general population (Daniels *et al.*, 1995; Warren *et al.*, 1992). We measured the ability of the HVR-3-like peptide epitopes (*dna* J from *E. coli* and gp110 for EBV) to induce T cell proliferation in autistic and normal subjects. Both *E. coli dna* J and EBV gp110 are significant antigens of each pathogen and *dna* J antibodies and activated T cell clones have been reported in the synovial fluid of RA patients (Winfield, 1989; Albani *et al.*, 1992a; Ikeda *et al.*, 1996). Our results failed to provide support for the hypothesis that DR4- or DR1-postitive autistic individuals are tolerant to the *E. coli* and EBV peptides that share sequences with the HVR-3 of HLA DR4 or DR1. The same caveat holds in this study of the response to HLA DR4- and DR1-like peptides as for the investigation of the response to pathogen-derived peptides: a comparison of group averages will not detect a small number of cases of autism that result from immune tolerance of *E. coli* or EBV in DR4- or DR1-positive subjects.

Although peptide-specific differences in T cell response between autistic and normal subjects were not detected in this investigation, a striking finding was the generally lowered T cell proliferation seen in autistic individuals. The analysis of

peptide-specific responses was made by comparisons of the SI. However, when the absolute amount of ^{3}H-thymidine incorporated in the assay is compared, the PBMC of autistic individuals incorporated only about half the amount of ^{3}H-thymidine with or without peptide than PBMC of normal subjects. This difference could be due to reduced T cell activity in autistic individuals as the same numbers of fresh PBMC (5×10^{6}) were assayed in all individuals. It is likely that reduced T cell function would adversely affect the immune capabilities of autistic subjects. A generally depressed immune response using various measures has been reported frequently in autism (Warren *et al.*, 1987, 1990, 1991; Yonk *et al.*, 1990). This global depression in immune responsiveness, whatever its cause, may play a more important role in autism than reduced responsiveness to any one particular pathogen.

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CHAPTER 5

SUMMARY

Immune system abnormalities and infection have long been suspected as factors that trigger autism (Rimland, 1964; Warren *et al.*, 1986). However, before the work reported here, it was unknown whether T or B cell abnormalities played a dominant role in the disease, if there was expansion of specific T cell clones, or if autistic individuals responded abnormally to any specific pathogens. The involvement of T or B cells in autism was explored in this work by examining the expression of cytokine markers of T or B cell immune responses. Later studies examined if T cell clone expansion occurs in autism by assessing the expression of all 24 major T cell receptor (TCR) V-β chain genes in autistic and normal individuals. Finally, the potential link between T cell-mediated autoimmunity, infectious agents, and autism was explored by examining T cell proliferation in response to antigens derived from a panel of pathogens. The results of the studies reported here provide new insights into this complex disease.

My results demonstrating elevated levels of interleukin type 2 (IL-2) and interferon-γ in autism suggest that T cells rather than B cells are involved in the disease. Since IL-2 and interferon-γ are markers of CD4+ T cell activation, the elevated expression of these cytokines indicates that activated CD4+ T cells play a role in the disease. Activation of CD4+ T cells is also known as a Th1 type immune response and activation of B cells is termed a Th2 response. The finding of a Th1 immune response in autism is significant because treatment strategies for Th1 and Th2 responses differ. Therapy for Th1 responses involves administration of cytokines, such as IL-4, that are

elevated in Th2 type responses (Romagnani, 1995, 1996, 1997). This may be a productive avenue to explore for treatment of autism, a disease in which no effective treatments are available.

The TCR V-β chain repertoire was found to be altered in autistic patients, and this finding provided the clear evidence for the expansion of specific T cell clones. Using competitive reverse transcription-polymerase chain reaction (RT-PCR), I quantified the abundance of mRNA of each of the 24 major TCR V-β chains in autistic and normal individuals. This is the first time a study of this scope has been performed in the investigation of autism. As a group, autistic individuals express significantly higher levels of V-β 13 and lower levels of V-β 19 and 22. These results suggest that there is biased expression of specific T-cell clones and support the hypothesis of altered T cell function in autism.

The antigen-presenting human leukocyte antigen (HLA) DR4 or DR1 allele occurs frequently in autistic individuals. I examined the possible association between these HLA DR molecules and T cell clone expansion by searching for an association between these alleles and the expression of specific TRC V-β chains. HLA DR4- or DR1-postive autistic individuals (n = 7) showed significantly elevated V-β 13 expression compared to normal individuals (n = 5). This result highlights the role played by these HLA DR molecules in the disease. There was no difference in V-β 19 and 22 expression between the DR4- or DR1-positive normal and autistic individuals. It appears that the expansion of V-β 13-expressing T cell clones is favored by HLA DR4 or DR1, whereas deletion of V-β 19 and 22 is independent of the HLA DR4 or DR1 alleles.

If the expansion of V-β 13 is a general feature of autism, it may open a path for the treatment of the disease. V-β 13-expressing clones could be blocked through immune therapy to potentially ameliorate disease symptoms. Moreover, the expansion of V-β 13-expressing clones may serve as the first physiologically based diagnostic tool for autism.

The final portion of this dissertation explored the possibility that infections by certain agents may contribute to autism. Epidemiological studies have linked infections and autism (Deykin and Macmahon, 1979). The hypothesis that autistic individuals demonstrate an altered immune response to some infectious agents was examined by observing T cell proliferation in response to a panel of T cell epitopes. These synthetic peptide epitopes represented the major T cell epitopes of a list of pathogens implicated in the development of autism. Comparison of a group of 24 autistic and 18 normal subjects did not show a reduced T cell proliferative response in autistic patients to peptides derived from rubella virus, herpes simplex virus type 1 (HSV-1), cytomegalovirus (CMV), or tetanus. The only difference between groups that was detected was an enhanced response to the influenza A virus epitope (matrix (M) protein) in autistic subjects. Therefore, these results do not provide support for the idea that a lower immune response to infectious agents plays a significant role in the disease. It is important to realize, however, that comparisons were made between the group averages of autistic and normal subjects and that widely varying individual responses were seen in each group. It remains possible that some cases of autism are caused by infectious agents, as suggested by previous epidemiological studies. However, if these cases occur at low frequency, they would be undetected because they would not significantly affect the average group

response. If autism indeed is a multifactorial disease, teasing out any individual factor becomes difficult.

One of the most striking findings in the immunogenetics of autism is the strong association of HLA DR4 or DR1 with the disease. One postulate for this association is molecular mimicry between these antigen-presenting molecules and sequences found in some common pathogens. The *Escherichia coli* (*E. coli) dna* J protein and EBV gp110 are both significant antigens of these infectious agents (Roudier *et al.*, 1988, 1989; Ikeda *et al.*, 1996) and both possess an amino acid sequence that is nearly identical to a critical region of the DR4 or DR1 gene products.

Results reported in Chapter 4 of this dissertation failed to provide support for the hypothesis of molecular mimicry between HLA DR4 or DR1 and *E. coli dna* J or EBV gp110. No relationship between possession of the HLA DR4 or DR1 and the ability to respond to the *E. coli* and EBV epitopes was detected in any group examined. There were no differences in response to these peptides related to the DR4 or DR1 allele. The same caveat holds in this study of the response to HLA DR4- and DR1-like peptides as for the investigation of the response to pathogen-derived peptides: a comparison of group averages will fail to detect a small number of cases of autism that result from immune tolerance of *E. coli* or EBV in DR4- or DR1-positive subjects.

What do the results of this dissertation say about the etiology of autism? First, it is essential to realize that autism is likely to be a multifactorial disorder; no one cause is responsible for autism in all affected individuals. Two leading postulates for causative factors in autism are autoimmunity and infectious agents. My results provide support for a role of autoimmunity in the disease, but failed to find evidence for the influence of

specific pathogens. Activation of CD4+ T cells is a hallmark of T cell-mediated autoimmunity. Therefore, my observation that many autistic individuals have activated CD4+ T cells suggests the involvement of autoimmunity in the disease. Significantly, there appears to be an expansion of specific T cell clones in autistic individuals. This was demonstrated by the augmented expression of one V-β 13 mRNA. A similar situation occurs in the autoimmune diseases rheumatoid arthritis and multiple sclerosis where mono- or oligoclonal T cell expansion is observed (Urban *et al.,* 1988; Jenkins *et al.,* 1993). There are many models that can incorporate these observations into the etiology of autism, but at this stage, all are speculative. The proposal I favor merges the results of this dissertation with the observation that in animal models activated T cell clones in peripheral blood can cross the blood-brain barrier (Wekerle *et al.*, 1986). I propose that activated peripheral blood T cells cross the blood-brain barrier where they recognize CNS components as antigens. These activated T cells are predicted to release IL-2 and other cytokines that recruit inflammatory cells, macrophages, effector T cells, and antigen presenting cells. These cells would produce CNS tissue damage. In combination with autoimmune attack on the CNS, this would create CNS lesions that cause autism. In support of these ideas, in animal models of multiple sclerosis, specific T cell clones activated in peripheral blood are also found within CNS tissues.

Obvious questions that stem from this model is whether T cell infiltration can be demonstrated in autism and, if so, whether T cell infiltration is involved only in autism or plays a more general role in other neurological disorders. An animal model of autism, which is currently not available, would greatly accelerate research into these and related questions.

Both Todd (1986) and Warren *et al.* (1987) proposed immune tolerance as an alternative mechanism to autoimmunity in the etiology of autism. Autistic individuals often exhibit decreased immune functions of T cells (Warren *et al.*, 1986), B cells (Warren *et al.*, 1986), natural killer cells (Warren *et al.*, 1987), immunoglobulin A (Warren *et al.*, 1997), and complement C4B (Warren *et al.*, 1995). My results did not indicate a reduced T cell proliferation in response to any single peptide in autistic individuals, but did show that there was a generally lowered T cell proliferation in autistic subjects whether untreated or challenged with peptide. It is likely that reduced T cell function would adversely affect the immune competence of autistic subjects. This widespread reduction in immune responsiveness, whatever its cause, may play a more important role in autism than immune tolerance to specific pathogens. Given the proposed mulitfactorial nature of autism, autoimmunity and a generally depressed immune system function are not mutually exclusive possibilities for the development of the disease.

The results of this dissertation open more questions than they answer and a comprehensive understanding of the disease remains a distant hope. Some of my results, however, especially the finding of specific T cell clone expansion, may pave the way to diagnosis and, more optimistically, therapy for the disease.

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APPENDICES

APPENDIX A. PHA STIMULATION OF CRYOPRESERVED PBMC: A METHOD TO AMPLIFY T CELLS

APPENDIX A

PHA STIMULATION OF CRYOPRESERVED PBMC: A METHOD TO AMPLIFY T CELLS

ABSTRACT

An important technical issue in the analysis of T cell populations involved in human T cell-mediated diseases is the limited number of these cells in affected tissues (Fujihashi *et al.*, 1996; Jenkins *et al.,* 1993). Amplification of primary T cell populations by mitogen treatment is a commonly used approach to circumvent this problem. Often, the primary populations of T cells are cryopreserved prior to mitogen stimulation. However, there are conflicting reports regarding whether amplification of primary T cell populations skews the distribution of T cell subtypes. In this communication, the affect of phytohemagglutinin (PHA) stimulation of freshly isolated or cryopreserved peripheral blood mononuclear cells (PBMC) on the distribution of T cell subtypes was examined. T cell receptor (TCR) V-β chain expression was used to assess the distribution of T cell subtypes. The expression of all the 24 major TCR V-β chains was analyzed by competitive reverse transcription-based polymerase chain reaction (RT-PCR) in the PBMC of three subjects. The results show that PHA stimulation of fresh or cryopreserved PBMC did not significantly alter the TCR V-β chain repertoire of the cells.

INTRODUCTION

Analysis of TCR repertoires is used to identify T cell clones that are activated during infection and disease (Ohga *et al.*, 1999; Than *et al.*, 1999). The expansion of a single or limited number of T cell clones occurs in many human autoimmune disorders

(Wucherpfennig *et al.*, 1995; Abe *et al.*, 1993; Moller *et al.*, 1988). The identification of these clones offers the hope of therapy based on deletion of the specific activated clones (Urban *et al.*, 1988; Vandenbark *et al.*, 1989). The variable region of the β chain (V-β) is the most commonly examined molecular marker of T cell identity (Arche-Orbea *et al.*, 1988; Keystone *et al.*, 1995; Posnett *et al.*, 1990). The small number of T cells that can be recovered from either affected tissues or the peripheral blood is a limiting factor in the study of autoimmune and other T cell-based disorders. One approach to circumvent restricted T cell number is to amplify the T cell population by treatment with a mitogen such as PHA. A concern with such an approach is that particular clones may be selectively amplified or lost, creating an altered population that poorly mirrors the original clonal diversity. There are conflicting reports as to whether this is a real or hypothetical issue. Using flow cytometry and a limited list of monoclonal antibodies available for TCR V-β chain, Davey and Munkirs (1993) found that treatment of PBMC with the mitogen ConA skewed V-β chain expression compared to the starting population of cells. However, when using PHA to stimulate growth, Jason *et al.* (1996) found that there was an unbiased representation of clones in the expanded population.

The studies reported in Chapter III of this dissertation required a significant number of T cells from a large group of autistic and control individuals. This necessitated some method of increasing T cell number. The studies presented in this appendix examined if either of two methods of T cell expansion produced an unbiased V-β repertoire identical to that of the initial T cells. The expression of the 24 major V-β chains was studied using the sensitive technique of competitive RT-PCR. The results

show that PHA stimulation of either fresh or cryopreserved PBMC did not alter the repertoire of V-β chain expression. These results justify the use of PHA-stimulated fresh or cryopreserved cells in the studies of this dissertation.

MATERIALS AND METHODS

Isolation of Total RNA

Blood was drawn from three volunteers into vacutainer tubes and PBMC were isolated by histopaqua (Sigma, St. Louis, MO) density centrifugation. The PBMC were divided and placed into three treatment groups: 1) fresh PBMC (5×10^6); 2) fresh PBMC (5×10^5) cultured with 10 μg/ml PHA (Gibco BRL, Grand Island, N.Y.) for five days; and 3) PBMC (5×10^5) cryopreserved at -70°C for 10 days, thawed, then stimulated with PHA as above. Total RNA was isolated using Trizol (Gibco, Gaitherburg, MD) from 5×10^6 primary or PHA-stimulated PBMC, and then dissolved in 30 μl of sterile H_2O. Total RNA concentration was measured by absorbance at 260 nm.

cDNA Synthesis and RT-PCR

To synthesize cDNA, 2 μg of total RNA was reverse-transcribed in a 20-μl reaction mixture at 37°C for two hours according to the manufacturer's instructions (Promega, Madison, WI). The resulting cDNA was divided equally into 24 tubes, each containing a 5' TCR V-β-specific sense primer, a common 3' TCR V-β C antisense primer, 100 femtomoles of a reference cDNAs (also called competitor), and the other PCR components described in Chapter III of this dissertation. PCR was performed in a 15 μl reaction volume using an initial 5 minutes of denaturation at 94 °C followed by 30

cycles of the profile: 1 minute at 94 °C, 1 minute at 58 °C and 1 minute at 72 °C. TCR V-β 21 was used to optimize competitive RT-PCR, and these conditions were applied to all 24 V-β chains. PCR products were analyzed by electrophoresis through 2% agarose gels.

Analysis of PCR Products

Analysis of PCR products was performed according to Spinella and Robertson (1994). Briefly, a photograph of the agarose gel was converted to a digital image. The image was analyzed using the digital processing program from Biosoft Inc. (Ferguson, MO). The intensities of endogenous cDNA products were normalized relative to that of the most intense band. A similar normalization was performed for the reference cDNA amplification products, each 86-bp larger than the corresponding endogenous bands. The ratio between the normalized values of each endogenous band relative to its corresponding reference band was calculated and termed the relative expression index (REI).

Statistical Analysis

The V-β repertoires of each treatment regimen were arranged in descending order of REI values. For each treatment in each individual, Spearman's rank test was used to calculate an r_s value [$r_s = 1 - (6 \times (\sum d_i^2)/(n^3-n))$], where di represents the rank difference between the repertoires of fresh PBMC vs the repertoires of any other two treatments, and n is the sample size. Then, Spearman's t test was employed according to Harnett (1982) to test the significance of differences between treatments. In Spearman's t test, t=

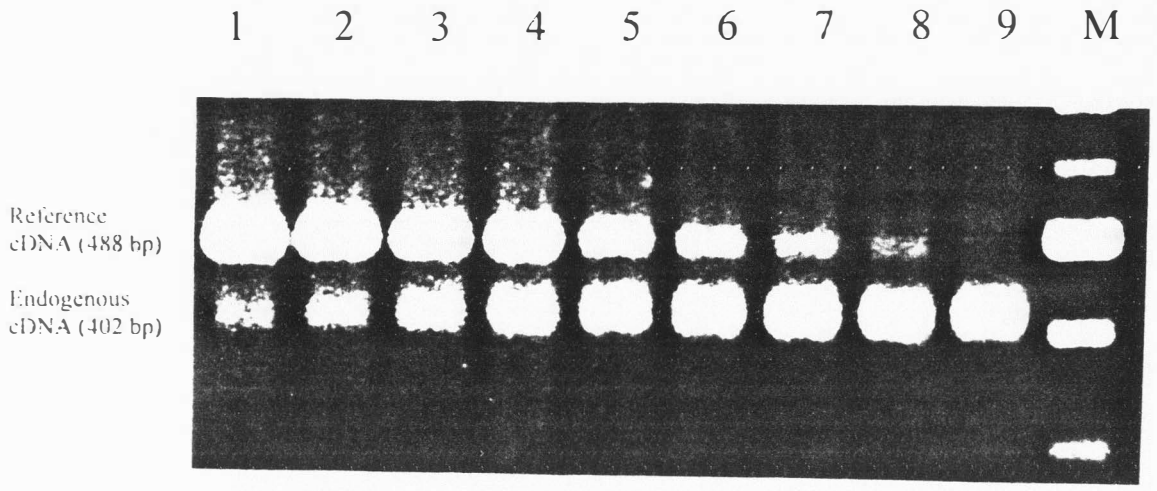
$r_s \times [(n-2)/(1-r_s^2)]^{1/2}$, while r_s represents the rank sum and n is the sameple size. The t value was used to assess the significance of differences (p value).

RESULTS AND DISCUSSION

Analysis of V-β Chain Repertoires by Competitive RT-PCR

V-β chain expression was analyzed by competitive RT-PCR. This technique has the advantages of sensitivity relative to Southern analysis and flow-cytometry, two methods traditionally employed for these studies. Optimizations of RT-PCR were performed using V-β-21 as a representative V-β chain. Two μg of total RNA from PBMC were used to synthesize cDNA. Aliquots (2 μl) of the cDNA were co-amplified with varying amounts of reference cDNA. Reference cDNA is a V-β-21 gene modified by insertion of an 86-bp sequence. This distinguishes reference and endogenous cDNA amplification products. Amplification was carried out for 30 cycles. The amount of starting endogenous cDNA is determined in one of two ways. In an experiment, such the one shown in Figure 6.1, the lane that demonstrates equal intensity of the reference and endogenous cDNA bands gives the amount of starting endogenous cDNA as this equals the known starting amount of reference cDNA. In Figure 6.1, this is lane 5 and the amount of endogenous cDNA therefore is 25 femtomoles. In large-scale assays involving many samples, such as those used to analyze the 24 major V-β chains from dozens of individuals, optimization for all samples is impractical. In this case, one takes advantage of the linear relationship between the log ratio of reference/endogenous band intensity to the log of reference cDNA molecules. This relationship for the PCR shown in panel A of

A.



B.

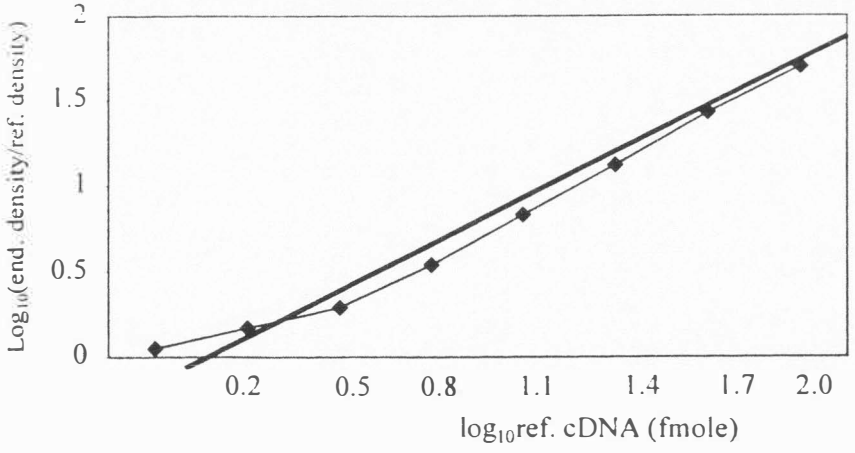


Fig. 6.1. Competitive RT-PCR analysis of TCR V-β 21 expression. Panel A. Two μl of the endogenous cDNA were co-amplified with 2 μl of reference cDNA prepared as a series of 1:2 dilutions with a starting amount of 100 femtomole (1 fmole = 10^{-15} moles) (lane 1-8). Lane 9 contained 2 μl of endogenous cDNA only. M: DNA size marker. Panel B. Quantitative analysis of the data plotted in panel A.

Figure 6.1 is demonstrated in panel B of the same Figure. In experiments, such as those presented in Chapters II and III, the $\log_{10}$-transformed ratio of reference to endogenous bands is determined and the amount of endogenous cDNA is derived from the x intercept of this ratio.

Having established conditions for the amplification of TCR V-β-21, the assay was extended to all 24 V-β clones. Two μl of endogenous cDNA and 10 femtomoles of reference cDNA were aliquoted into 24 tubes and amplified by PCR under identical conditions. Figure 6.2 shows a gel displaying a typical PCR using cDNA derived from fresh PBMC of a single donor. In each lane, two DNA bands were generated, the smaller one from endogenous cDNA and the larger one from the reference cDNA. As expected, the products from reference cDNAs were 86-bp larger than their corresponding endogenous products.

Analysis of the relative levels of expression of each V-β chain was performed using software from Biosoft, Inc. The analysis compares the densities of the endogenous and reference cDNA products in each lane and normalizes these relative to the highest endogenous/reference cDNA ratio observed in an individual. These normalized ratios, termed the relative expression index (REI), assess the relative expression level of each V-β chain in an individual. Analysis of the PCR shown in Figure 6.2 indicated that V-β 5, 8, and 13 were the predominant V-β chains expressed in this individual (Figure 6.3).

Comparison of the TCR V-β Repertoire from Fresh and Amplified PBMC

V-β chain expression was compared in fresh PBMC, fresh PBMC directly

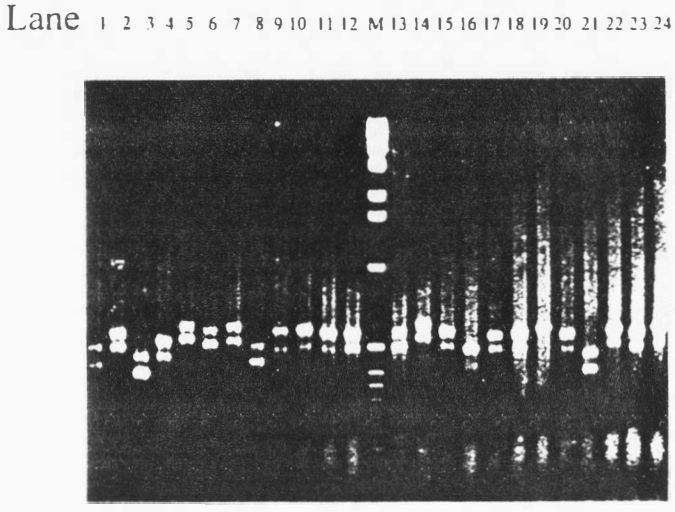


Fig. 6.2. RT-PCR of 24 TCR V-β mRNAs expressed in PBMC of donor 1. The total RNA was extracted from peripheral blood mononuclear cells and cDNA was synthesized in vitro using reverse transcriptase at 37°C overnight. The resulting cDNA was used as the template to set up PCR. PCR Then, the PCR products were run on 2% DNA agarose gel for 1 hour, stained by ethidium bromide and visualized under UV light. (Lanes 1-12 represent TCR V-β 1-12; Lane M representsl-kb DNA size marker and 500-bp DNA size band is the major marker to judge the RT-PCR products; Lanes 13-24 represent RT-PCR products from TCR V-β 13-24.) All RT-PCR products were identified to have the correct sizes and the endogenous products were confirmed to be constantly 86-bp smaller than the corresponding reference cDNA products.

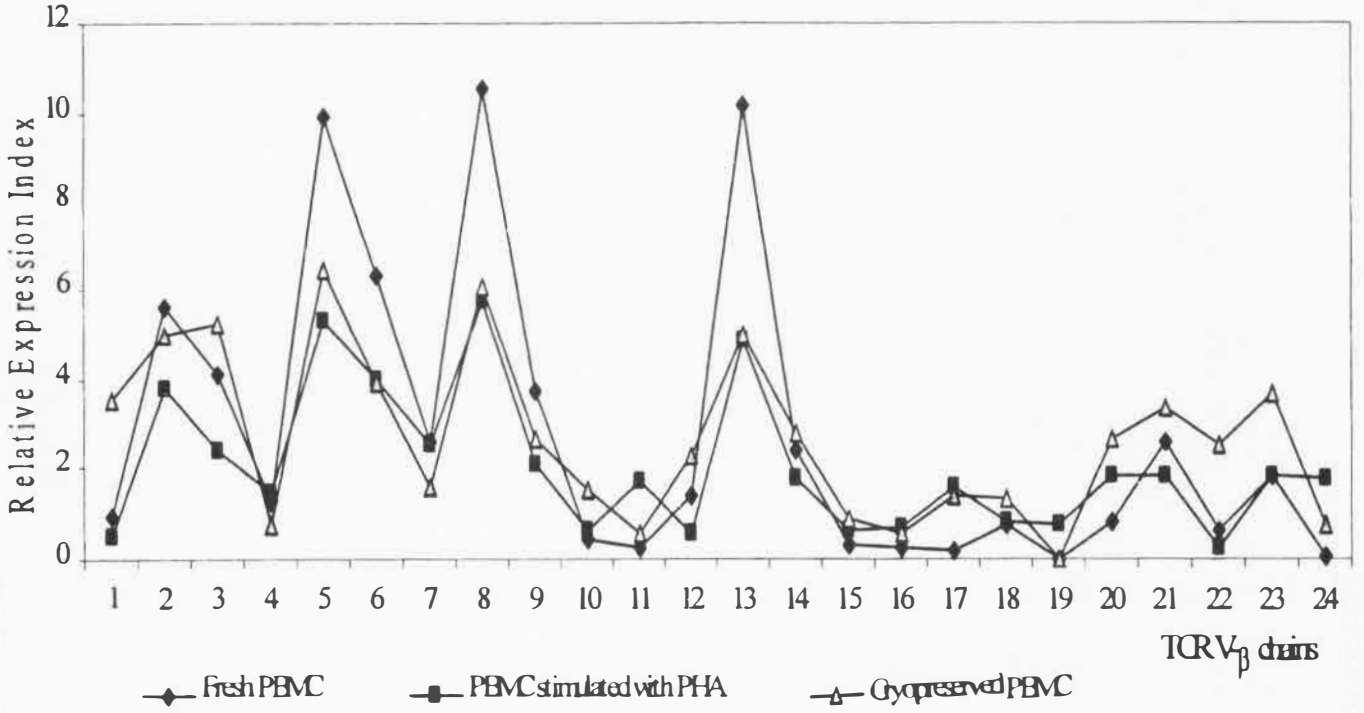


Fig. 6.3. TCR V-β repertoire trends in three PBMC preparations from donor 1.

stimulated by PHA, and cryopreserved PBMC stimulated by PHA after thawing. Figure 6.3 shows the REIs of 24 V-β chains from fresh or PHA-stimulated PBMC taken from the same individual analyzed in Figure 6.2. This analysis shows that V-β 2, 5, 8, and 13 were the predominant clones in all three PBMC preparations. Visual inspection of the results indicates that PHA treatment alone or coupled with cryopreservation did not alter the V-β repertoire. PBMC preparations from two additional donors were treated under the same three conditions (Figures 6.4 and 6.5). Once again, visual inspection showed that the expression repertoire was not altered by PHA treatment alone or PHA treatment following recovery from cryopreservation. A different pattern of V-β chain expression was observed in each of the three normal subjects (Figures 6.3, 6.4 and 6.5).

A more rigorous analysis of the data was made using Spearman's rank test. This test measures the significance of differences in rank of V-β chain expression under the three experimental conditions. A p value of less than 0.01, which indicates no significant difference, was found in each subject for comparisons between V-β expressions in fresh PBMC relative to PBMC stimulated by PHA under either treatment regimen (see Table 6.1).

These findings of the study are significant because they show that long-term storage of T cell populations and their subsequent amplification is possible without skewing the original composition of T cell subtypes.

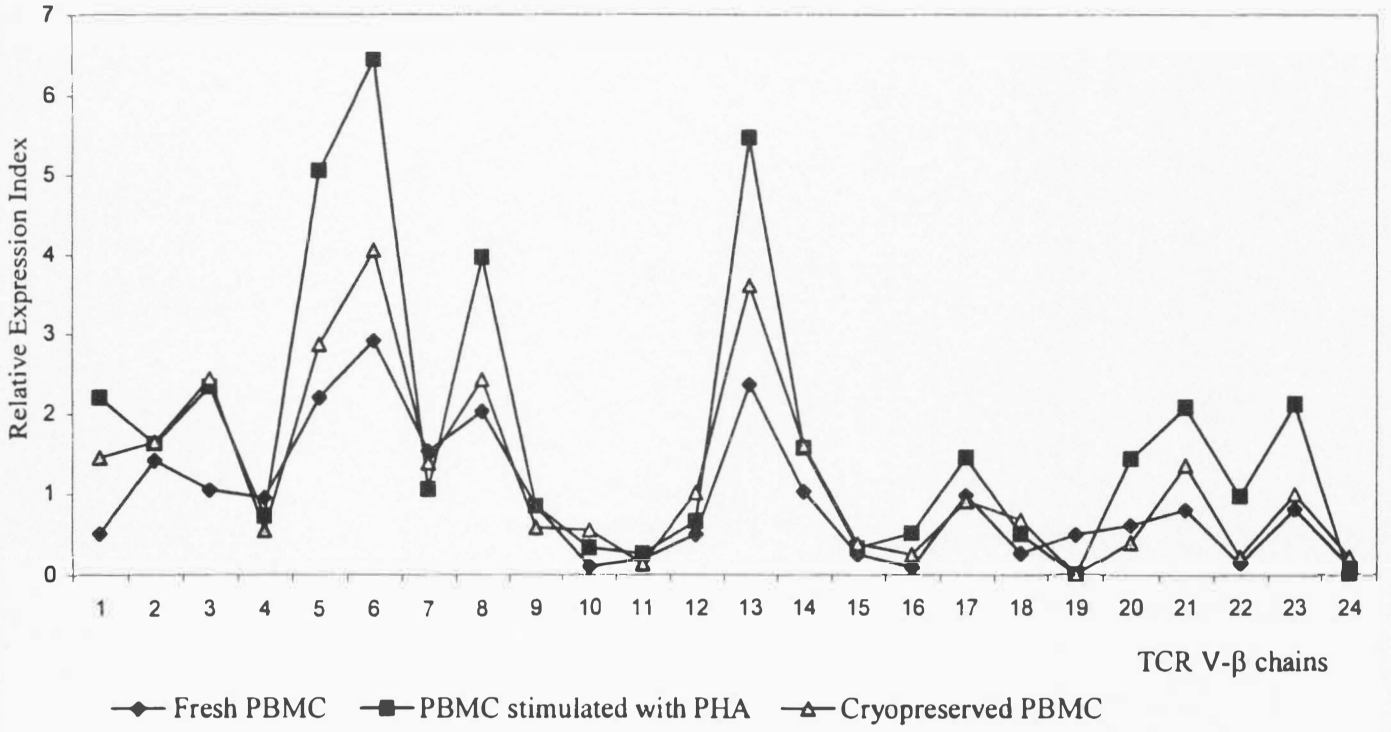


Fig. 6.4. TCR V-β repertoire trends in three PBMC preparations from donor 2.

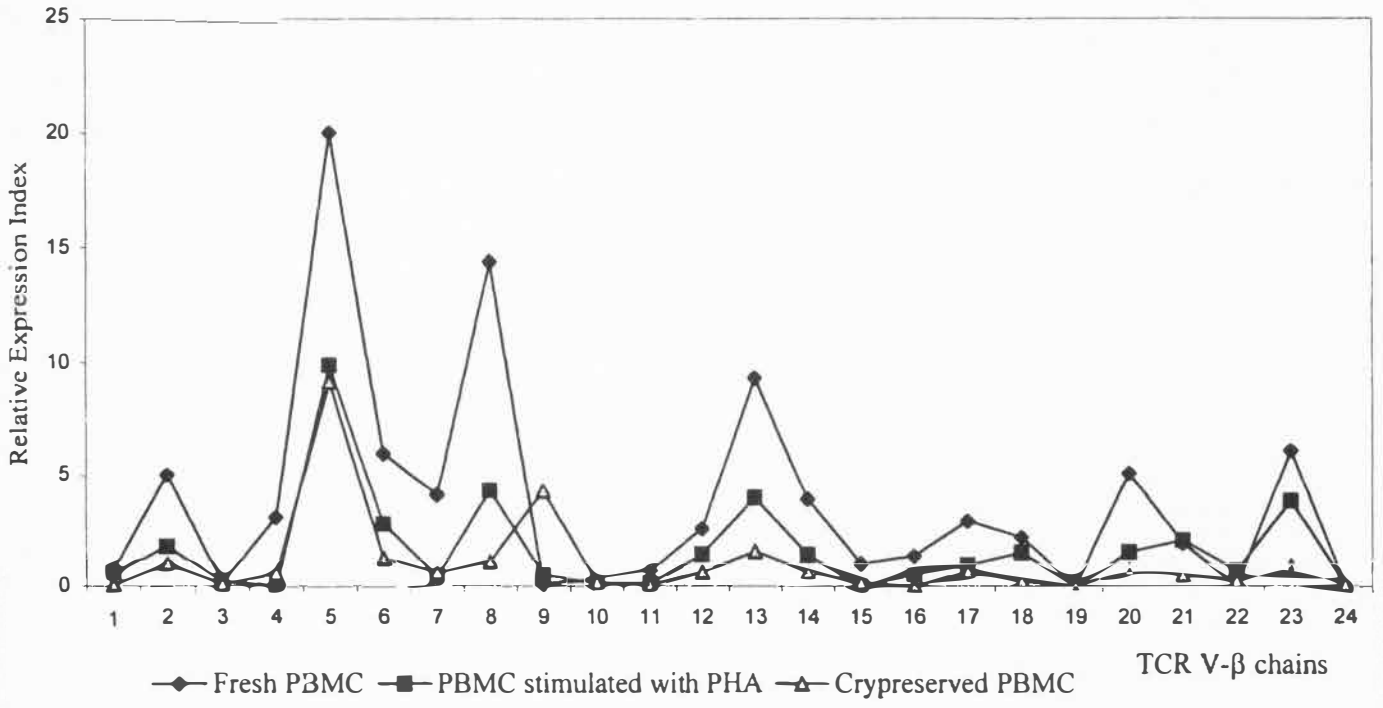


Fig. 6.5. TCR V-β repertoire trends in three PBMC preparations from donor 3.

Table 6.1
Spearman's Rank Test of TCR V-β Expression in Three Donors

Donor	Fresh vs PHA-Stimulated			Fresh vs Cryopreserved		
	r_s[a]	t[b]	p[c]	r_s[a]	t[b]	p[c]
1	0.766	5.59	< 0.01	0.744	5.23	<0.01
2	0.813	6.56	<0.01	0.849	7.55	<0.01
3	0.774	5.73	<0.01	0.884	8.89	<0.01

[a]The rank sum values of Spearman's rank test.
[b]t values of Spearman's rank test.
[c]p value of Spearman's rank test.

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APPENDIX B. OPTIMIZATION OF A PEPTIDE-MEDIATED T CELL PROLIFERATION ASSAY

APPENDIX B

OPTIMIZATION OF A PEPTIDE-MEDIATED T CELL PROLIFERATION ASSAY

ABSTRACT

Optimal conditions were established for T cell proliferation stimulated by synthetic peptide epitopes. The effect of varying the epitope concentration, the type and amount of cytokines, and the concentration of serum were examined. The optimized assay utilized peripheral blood mononuclear cells (PBMC) cultured with 10% fetal bovine serum, 30 international units (IU)/ml interleukin type 2 (IL-2), and 1 μM synthetic peptide.

INTRODUCTION

The T cell proliferation assay is an important experimental tool to assess the response of T cells to antigens (DuPont *et al.*, 1985; Rychlikova *et al.*, 1971; De Graeff-Meeder *et al.*, 1991). Current applications of the assay often employ synthetic peptide-mediated epitopes to stimulate T cell proliferation (Schrader *et al.*, 1991). It is critical to establish optimal conditions for the assay, especially when using synthetic epitopes, which often are only weakly antigenic (Bromelow *et al.*, 1997; Yang *et al.*, 1995; Durinovic-Bello *et al.*, 1996). The influence of peptide concentration, the type and amount of cytokines, and serum levels on the T cell proliferation assay were investigated (Bertram *et al.*, 1997; Hao *et al.*, 1986; and Lamers *et al.*, 1992).

MATERIALS AND METHODS

Synthetic Peptide Epitopes

Ten peptides derived from known or predicted epitopes of pathogens potentially involved in autism were synthesized at the Utah State University Biotechnology Center. The peptides were purified by reverse-phase high performance liquid chromatography, and diluted in RPMI-1640 (RPMI Inc., Cellgro, VA). Their amino acid sequences are listed (see Table 4.1 of chapter IV).

T Cell Proliferation Assay

PBMC were isolated from fresh blood of normal healthy donors by histopaque (Sigma, St. Louis, MO) density centrifugation. PBMC were cultured in 96 well plates using RPMI-1640, fetal bovine serum (usually 10%), and synthetic epitopes. When recombinant cytokines were included, they were added 24 hours after beginning cell culture (Chain *et al.,* 1987). Five days after adding synthetic peptide, 2 μl of ^{3}H-thymidine (1 μCi; Moravek Biochemicals. Inc., Brea, CA) was added to each well, cells were incubated for 3 hours, then transferred to a filter membrane (Packard Instrument Inc., Dower Grove, IL) using a cell harvesting device (Micromate-196, Packard Instrument Inc., Dower Grove, IL). The unincorporated ^{3}H-thymidine was removed by vacuum filtration, and the counts associated with the cells determined using a direct β-ray counter (Matrix^{TM}-96, Packard Instrument Inc., Dower Grove, IL). Proliferation indices were expressed as the ratios of the average counts per minute (cpm) from triplicate antigen-containing test wells to the average cpm of triplicate control wells without antigen.

RESULTS AND DISCUSSION

Peptide Concentration

The effect of 4 different synthetic peptides, rubella capsid, rubella E2, HSV-1 gc-1 and tetanus toxin on T cell proliferation was investigated in cells cultured without any added cytokines. Each peptide was added at 5 different concentrations that ranged between 1 x 10^{-3} μM – 10 μM. Typical peptide concentrations used in T cell proliferation assays range between 0.01 and 1 μM (Orlik *et al.*, 1996; Mayer *et al.*, 1996). The proliferation index at any peptide concentration never exceeded 1.6. Later studies (see below) demonstrated that a cytokine must be added to the cells to increase the proliferation index to more significant values. Nonetheless, the peptide dose-response relationships shown in Figure 7.1 indicate that for most peptides a concentration of 1 μM was the most effective. Increasing the concentration to 10 μM almost always was inhibitory.

Cytokines

Cytokines play essential roles in T cell proliferation (Chain *et al.*, 1987). The effect of IL-2, interleukin type 3 (IL-3), interleukin type 4 (IL-4), interleukin type 6 (IL-6), interleukin type 12 (IL-12), platelet-derived growth factor (PDGF) on T cell proliferation without added peptide was investigated. As shown in Figure 7.2, IL-2 was the most potent cytokine for T cell proliferation. For peptide-stimulated T cell proliferation, a cytokine concentration that stimulates cell growth roughly between 2- and 10-fold is optimal. Increasing the proliferation index above these values produces a low

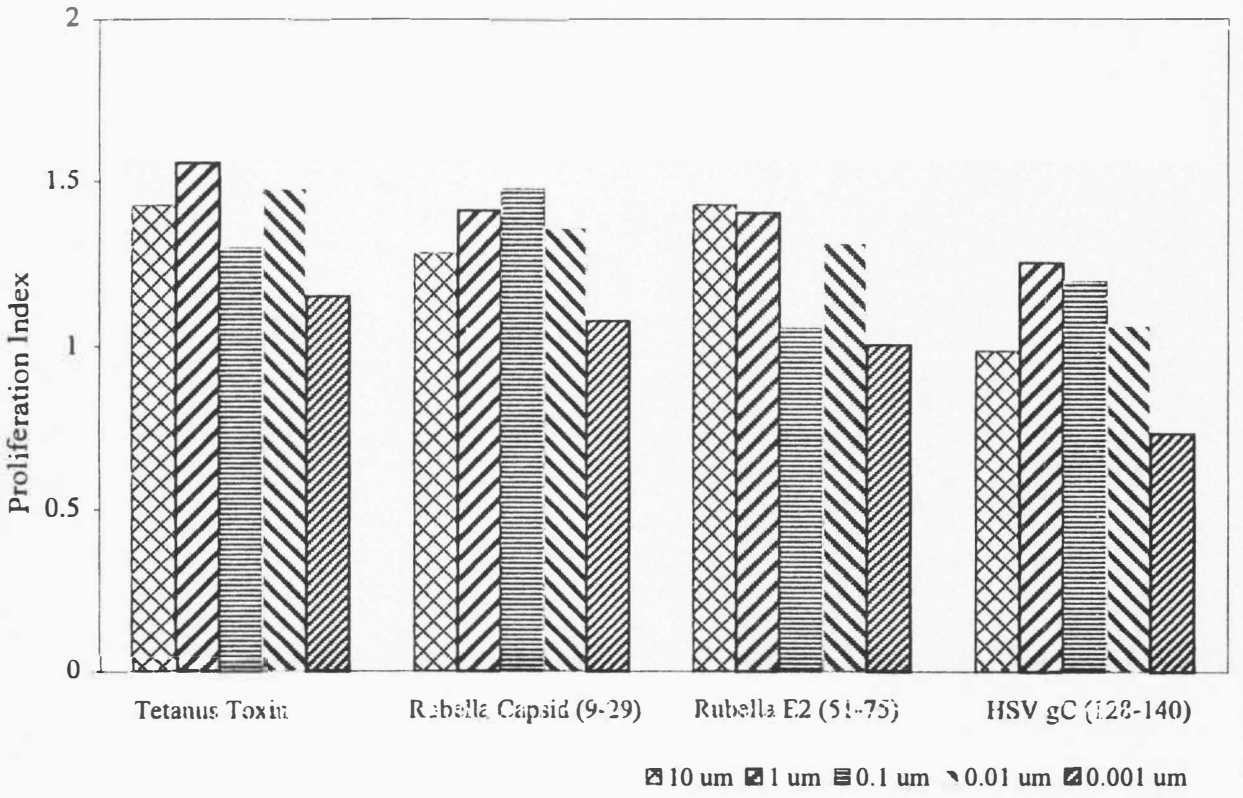

Fig. 7.1. Effect of peptide concentration on the T cell proliferation assay. Four synthetic peptides (Tetanus Toxin, Rubella Capsid (9-29), Rubella E2 (51-75), HSV gC (128-140), from left to right) were used. For each peptide, five concentrations (10, 1, 0.1, 0.01 and 0.001 μM, from left to right) were examined for their effects on the T cell proliferation assay.

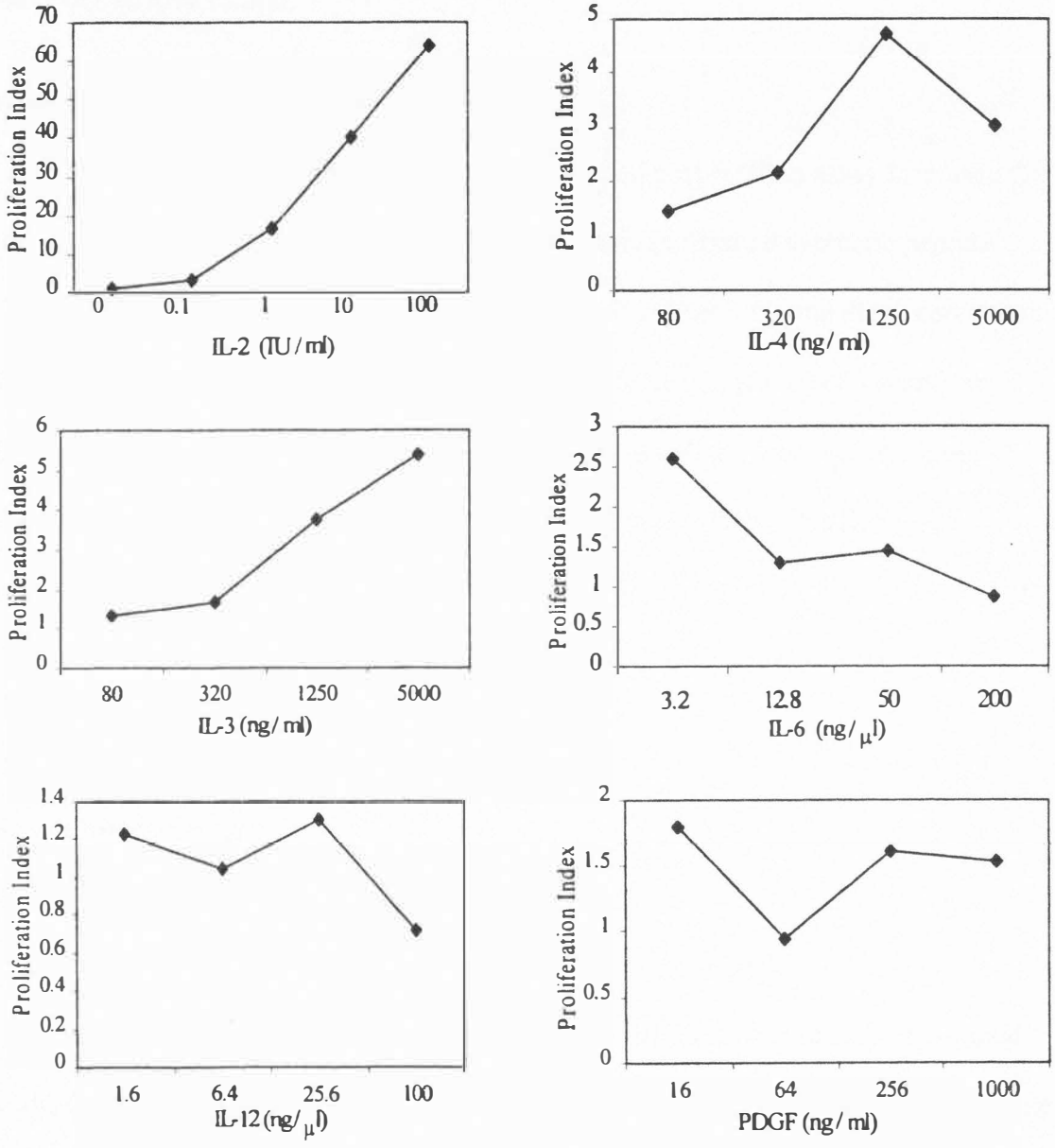

Fig. 7.2. Effect of different cytokines on the T cell proliferation assay.

signal-to-noise ratio associated with addition of peptide. For this reason, a concentration 30 IU/ml of IL-2 in the culture medium was chosen as the standard for assays of this dissertation.

Interaction between Peptide and IL-2

The effect of IL-2 addition (30 IU/ml) on T cell proliferation assay increased the sensitivity of the assay, and the stimulation index derived from 10 synthetic peptide epitopes used in this dissertation is shown in Figure 7.3. There is some disagreement on what constitutes a significant level of enhanced proliferation, with most researchers placing the minimum value of the proliferation index at either 1.5 or 2.0. Applying a value of 2.0, Figure 7.3 indicates that the peptide of Epstein-Barr virus (EBV) gp110 and tetanus toxin induced significant proliferation, but only when PBMC were cultured with IL-2. A significantly difference was found between with added IL-2 (30 IU/ml) and without added IL-2 by Student's t test ($p < 0.01$).

Serum Concentration

Published reports employing the T cell proliferation assays call for serum levels between 1-10% (Orlik *et al.*, 1996). The effect of fetal bovine serum concentrations of 1% and 10% on T cell proliferation stimulated with 3 different peptides was examined and the results are shown in Figure 7.4. A 10% serum concentration was essential for the assay.

The Optimized Assay

Based on the results described above, the standard T cell proliferation assay

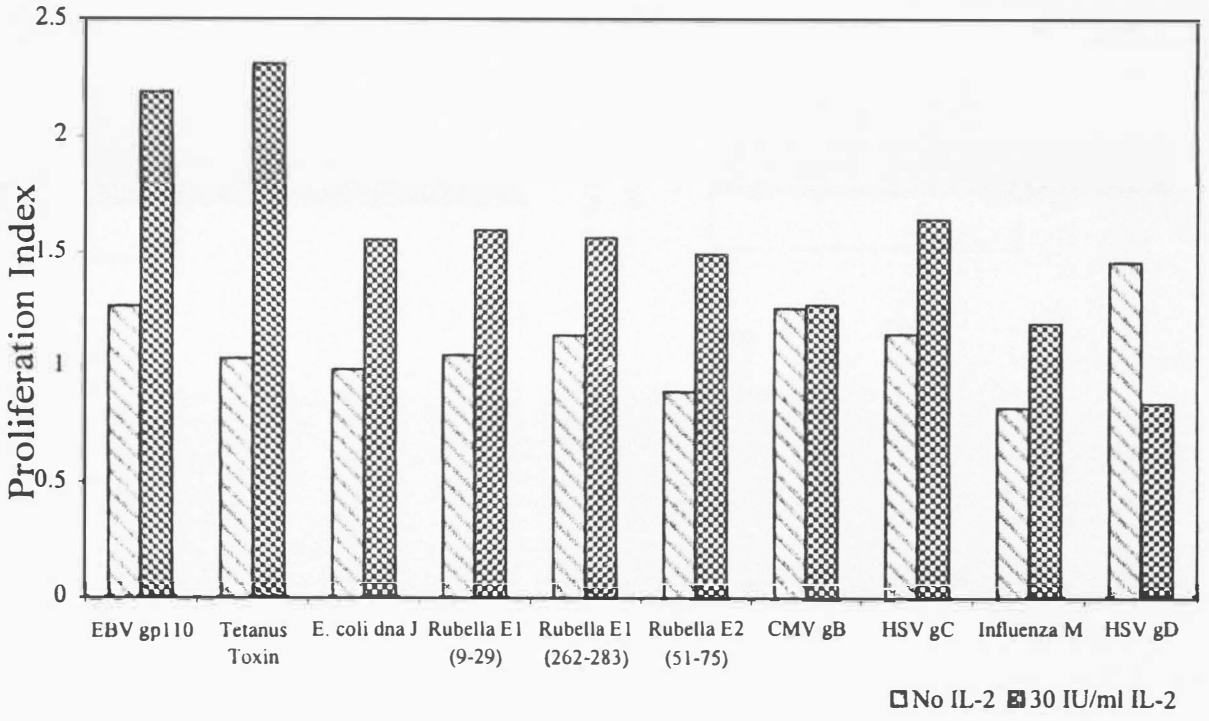

Fig. 7.3. Effect of IL-2 on the T cell proliferation assay. Ten peptides were tested with added IL-2 (30 IU/ml) or without added IL-2.

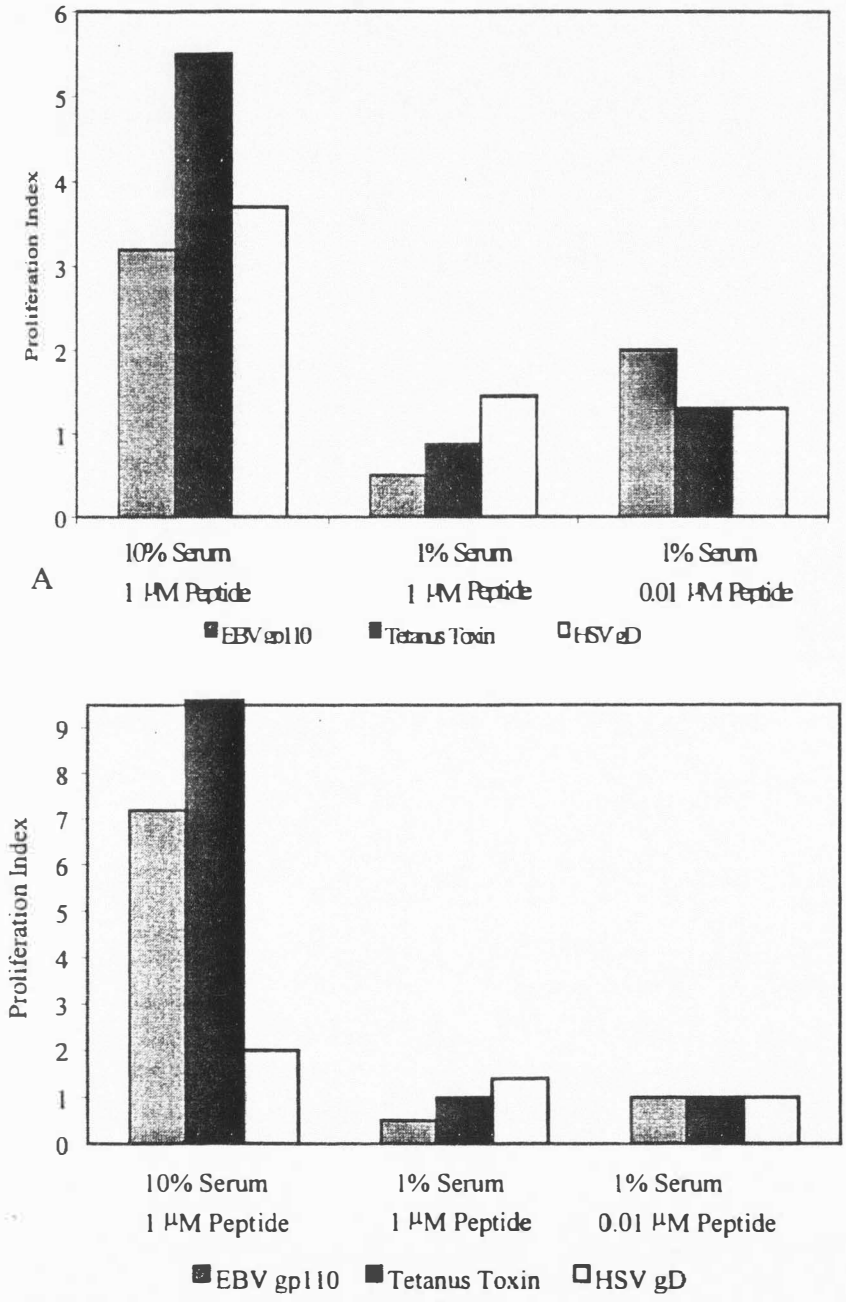

Fig. 7.4. Effect of varying serum and peptide concentrations on the T cell proliferation assay. Each graph panel (A or B) shows the results obtained from different individuals.

employed in this thesis utilized PBMC cultured with 10% serum, 30 IU/ml IL-2, and 1 μM synthetic peptide.

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